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# HEMATOPOIETIC AGENTS IN MACROCYTIC ANEMIAS[*]

ARNOLD D. WELCH AND ROBERT W. HEINLE

Departments of Pharmacology and Medicine, School of Medicine, Western Reserve University and University Hospitals, Cleveland, Ohio

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[*] Investigations conducted in these laboratories, referred to in this review, were supported, in part, by research grants from the National Institutes of Health, U. S. Public Health Service.

# HEMATOPOIETIC AGENTS IN MACROCYTIC ANEMIAS*

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**Departments of Pharmacology and Medicine, School of Medicine, Western Reserve University and University Hospitals, Cleveland, Ohio**

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## I. INTRODUCTION

In this review particular attention has been given to vitamin $B_{12}$, "intrinsic factor," folic acid and the hematologically active products of its metabolic alteration. Little or no space has been devoted to the many other substances which obviously are involved in hematopoiesis. Nor has any attempt been made to discuss all contributions to those fields which have been given attention. Although it was desired that an accurate picture of the present status of these hematopoietic agents be presented, it was necessary at the same time to give attention both to the limitations on space and to readability.

In referring to the factors with which we are concerned as *hematopoietic agents*, it may be pertinent to point out that these compounds are not limited in their function to processes concerned with the manufacture of cells found exclusively in the blood. Almost certainly both vitamin $B_{12}$ and folic acid, or their metabolically active derivatives, are essential constituents of a wide variety of the many forms of protoplasm. That they have come to be considered hematopoietic agents is related only to the fact that the cells of the blood normally must be regenerated at a rate incomparably greater than those of most other living tissues.

In view of the fact that the life of the human red blood cell averages only about 120 days (196, 278), it will be evident that a normal human subject must manufacture daily approximately 300,000,000,000 erythrocytes; in addition, a less well-defined but very great number of leucocytes must be formed. Since critical and essential roles are played by folic acid and vitamin $B_{12}$ in the synthesis of many key components of living cells, it is not surprising that either a deficiency of these factors or disturbances in their metabolic utilization often is manifested first by derangements of hematopoiesis.

## II. VITAMIN $B_{12}$ (CYANO-COBALAMIN)

### *A. Synopsis of historical background to the isolation of vitamin $B_{12}$*

So many reviews have been written concerning the etiology of pernicious and other megaloblastic anemias (60, 218, 385), the development of refined liver extracts for therapeutic use in the treatment of such anemias (327), and the development of vitamin $B_{12}$ (76, 100, 119, 175, 298, 315, 358) that only a brief synopsis for the sake of continuity and completeness is required in this article.

The effectiveness of whole liver, raw or lightly cooked, in the treatment of pernicious anemia was demonstrated by the well-known work of Minot and Murphy in 1926 (217), in which a daily dose of approximately one-half pound of liver usually was employed. However, within a relatively short time thereafter liver extracts suitable for intramuscular administration became available. At first these were quite crude (116, 324), but over a period of years more highly refined products (327) became available. Because no test animal proved suitable for the assay of the potency of liver extracts, in spite of numerous claims to the contrary (218), it was necessary that the activity of such preparations be determined by assay in patients with pernicious anemia in relapse, while potency was defined in terms of units (7). Experience proved that the monthly intramuscular administration of 30 units, or the administration at shorter intervals of enough material to supply an average of 1 unit daily, was sufficient to maintain a normal hematologic and, in most cases, normal neurologic status (322, 331). However, it has been customary to give larger, and undefined, doses to patients with severe or progressive neural involvement, because of the feeling that a larger amount than that necessary to maintain a normal hematologic status might be necessary to combat involvement of neural tissues (323). Liver extracts, like whole liver, have been effective when administered orally, although adequate therapy of pernicious anemia required that from 30 to 60 times the parenteral dose of relatively crude extracts be given (69, 70). Because of the necessity for large daily doses of rather unpalatable material, the oral administration of liver extracts has never been popular; in addition, some patients with pernicious anemia do not respond fully, even with large doses. It is not unlikely that the hematologic response to *orally* administered crude extracts of liver often was due to folic acid. This possibility will be discussed subsequently.

The active material in liver remained unidentified for many years, partly because the only test subject was the patient with pernicious anemia in relapse, and the small number of patients suitable for assay purposes greatly restricted the number of tests which could be carried out. With the discovery that the growth of certain species of lactobacilli is promoted by a substance present in the more highly refined liver extracts, as well as in whole liver and other substances (284), a rapid method for the assay of the active material became available; this finding led swiftly to the isolation of a crystalline compound, vitamin $B_{12}$ (255).

Almost simultaneously with the announcement of the isolation of the new vitamin by the American workers, there was reported independently by English investigators (292, 293, 295) the separation from liver of a compound which soon was proved to be identical with that isolated by Rickes and his associates (255). It would be inappropriate indeed not to give special recognition to the painstaking work of the British group, which, guided only by clinical assays, was able to concentrate the factor until an association between hematopoietic activity in patients and the appearance of a pink color (in still quite impure fractions) was suspected. Taking prompt advantage of this lead, Smith and his several colleagues (292, 293, 295) were able to crystallize the vitamin, without the aid of the rapid assays afforded by the use of microorganisms, and to announce their findings only a few weeks subsequent to the report of the American workers. Also, the presence of cobalt in the new vitamin was reported first by Smith (293).

### *B. Chemical nature of vitamin $B_{12}$*

Only a portion of the chemical structure of vitamin $B_{12}$ is known at the present time. In pure form it is a red crystalline material with certain well-defined physical and chemical characteristics (100); its chemical composition approximates $C_{61-64}H_{86-92}N_{14}O_{13}PCo$, with a molecular weight of about 1300. The largest fragment of the molecule to be identified so far consists of 5,6-dimethylbenzimidazole in glycosidic linkage with a molecule of ribose (at position 1), which in turn is phosphorylated at position $C_2$ or $C_3$. The single atom of cobalt is probably linked to the benzimidazole moiety (11, 12). In addition, acid hydrolysis causes the release of hydrogen cyanide (35) and the formation of two residues of aminopropanol (95). The latter substance was identified later as Dg-1-amino-2-propanol (388) and shown to be the fragment responsible for the ninhydrin reaction. Desoxyribose, purine and pyrimidine moieties do not appear to occur in the vitamin molecule (68). Certain degradation products of vitamin $B_{12}$, for instance, 5,6-dimethylbenzimidazole and 1,2-diamino-4,5-dimethylbenzene, exhibited vitamin $B_{12}$-like activity in rats when tested orally at milligram-levels; on the other hand, 2,5-dimethylbenzimidazole, under the same conditions, exerted a depressant effect on growth, a circumstance of possible significance to the development of antagonists of vitamin $B_{12}$ (99).

It was soon recognized that various forms of vitamin $B_{12}$ exist (292). Although the properties originally attributed to vitamin $B_{12a}$ by Kaczka *et al.* (179) and to vitamin $B_{12b}$ by Pierce *et al.* (244) suggested that these two forms of the vitamin might be quite different, it is now apparent that the difference actually is of little significance. In fact, vitamin $B_{12a}$ may be regarded as an anhydrous form of vitamin $B_{12b}$ (73). It was reported (179) that vitamin $B_{12a}$ had absorption maxima at 315, 352.5, 415 and 530 mμ and that its biological activity was significantly lower than that of vitamin $B_{12}$; thus, it had only 30 ± 15 per cent of the activity of vitamin $B_{12}$ in chicks, was only about one half as active in rats, and was about 20 per cent as active for *L. leichmannii*. A few weeks subsequently the crystallization of vitamin $B_{12b}$ from cultures of *Streptomyces aureofaciens* was announced by Pierce *et al.* (244). This compound differed from vitamin $B_{12a}$ in not having an absorption spectrum maximum at 315 $m\mu$ (244) and in having biological activity equal to that of vitamin $B_{12}$ in chicks and for *L. leichmannii* (245). It was shown by Brockman and coworkers (36) that it was possible to obtain a compound with the characteristics of vitamin $B_{12b}$, rather than those of vitamin $B_{12a}$, by hydrogenation of vitamin $B_{12}$ followed by oxidation with air. That both vitamin $B_{12a}$ and vitamin $B_{12b}$ can be converted into vitamin $B_{12}$ by treatment with cyanide was shown by various workers (35, 94, 347), although the first participation of cyanide in the formulation of vitamin $B_{12}$ was announced by Petrow (239).

Subsequent studies by Kaczka and associates (178) indicated that the absorption spectrum characteristic of vitamin $B_{12a}$ (with a band at 315 $m\mu$) was retained if vitamin $B_{12a}$ were dried at 100° for two hours *in vacuo* and the absorption spectrum determined immediately following solution of the product in water. However, upon standing the absorption spectrum changed to a pattern which was identical with that of vitamin $B_{12b}$. The authors also revised their earlier biological data and reported that vitamins $B_{12a}$ and $B_{12b}$ each have about one half the activity of vitamin $B_{12}$ in the chick, and that vitamin $B_{12a}$ and vitamin $B_{12}$ have comparable activity in the rat. A similar relationship between vitamin $B_{12b}$ and vitamin $B_{12}$ in the rat was reported by Hartman *et al.* (139). No explanation has been offered for the discrepancy between the findings of the Lederle group and the Merck group with respect to the activity of the derivatives, relative to vitamin $B_{12}$, in the chick. Differences in microbial activity (178) can be attributed to variations in assay procedure and to the degree to which the more reactive form of the vitamin, *i.e.*, that containing no cyano group (vitamin $B_{12a}$ or $B_{12b}$), was exposed to conditions under which it could have been altered chemically. More recently, vitamin $B_{12c}$ and vitamin $B_{12d}$ have been identified and subjected to chemical and biological studies (294).

Although adequate evidence has been presented to demonstrate that each of these compounds is markedly active in the treatment of pernicious anemia, the data available do not permit a conclusion to be drawn concerning their relative potency.

The material isolated originally and referred to as vitamin $B_{12}$ contains a cyano group which may be dissociated, *e.g.*, under the influence of light in weakly acid solution (35, 239, 347). Also, addition of potassium cyanide to the product was shown to reverse the reaction. Brink and associates (35) presented evidence indicating that the cyano group is bound coordinatively to the cobalt atom. It was proposed that the name, *cobalamin*, be assigned to all of the molecule of vitamin $B_{12}$ except the cyano group, and that the original material be termed *cyano-cobalamin* (180). According to this system of nomenclature, vitamin $B_{12a}$ and vitamin $B_{12b}$ are termed *hydroxo-cobalamin*, and vitamin $B_{12c}$ may be referred to as *nitro-cobalamin*. Many other forms of the vitamin may exist; thus, there have been produced, by substitution for the cyano group, such compounds as sulfato-cobalamin, chloro-cobalamin, and other derivatives. The reactivity of hydroxo-cobalamin in assay media during autoclaving, and the apparent presence in some materials of other forms of the vitamin which are convertible to cyano-cobalamin by treatment with cyanide, have led to the introduction of the pre-treatment of samples with sodium or potassium cyanide prior to microbial assay (32, 32a, 32b, 288, 298a).

### C. *Determination of vitamin $B_{12}$*

The content of cobalamin derivatives in various preparations is usually determined by microbial assay employing *Lactobacillus leichmannii*, either ATCC (American Type Culture Collection) 4797 or ATCC 7830; the latter sometimes is less specifically referred to as #313 (not an ATCC number). The original isolation procedures were guided by assays using a strain of *Lactobacillus lactis* (ATCC 8000), sometimes referred to as *L. lactis* Dorner; other organisms which have been used in assaying for vitamin $B_{12}$ are the chlorophyll-containing *Euglena gracilis* var. *bacillaris* (164), and strains of *Escherichia coli*. With the use of *E. coli* it is necessary to employ either mutant strains which cannot synthesize vitamin $B_{12}$ at a rate compatible with rapid growth (87) or a culture in which the synthesis of the vitamin is inhibited by the addition of a sulfonamide (279). In conducting assays with *Euglena gracilis* a very real disadvantage is encountered in that several days are required for adequate growth to occur; in contrast, appropriate lactobacilli (*e.g.*, ATCC 4797 or 7830) yield excellent growth in 18 to 24 hours.

The concentration of vitamin $B_{12}$, in purified media, required to produce half-maximal growth of each of these organisms is exceedingly low; with the lactobacilli it approximates 10 $\mu\mu$g. (0.000000010 mg.) per ml. (175). For ATCC 7830 the sensitivity has been reported to be somewhat less; however, the difference is not marked and in many laboratories this organism is given preference for assay purposes.

Since the role of vitamin $B_{12}$ in bacterial metabolism is more conveniently treated under mechanism of action, this aspect of the vitamin will be discussed in a later section. Pertinent to the problems of determination of the various forms of vitamin $B_{12}$, however, are the possible errors to be encountered if certain compounds are present in materials to be assayed. Thus, the desoxyriboside of thymine, thymidine, rather specifically obviates the requirement for vitamin $B_{12}$ of several of the lactobacilli, but not for *Euglena gracilis* (164) or *E. coli* (87). With some species of lactobacilli, any of the purine or pyrimidine desoxyribosides will abolish the requirement for the vitamin. This subject is discussed more fully in the review by Jukes and Stokstad (175). From the practical standpoint, when assays are carried out with lactobacilli on materials rich in desoxyribosides, it may be necessary to compare the activity of one aliquot with that of another suitably treated with alkali to destroy the vitamin $B_{12}$. Autoclaving the materials to be assayed at pH 10 for 1.5 hours usually is sufficient to inactivate the vitamin, but does not influence the microbial activity of thymidine (159a, 367). Another problem that contributes to the difficulties of obtaining reliable results from microbial assays is the influence of such reducing agents as ascorbic acid on the apparent activity (298). It has been found desirable to add ascorbic acid, thiogylcolic acid, thiomalic acid, or some other reducing agent, to protect vitamin $B_{12}$ from destruction during autoclaving of assay media (298). It has been shown that "vitamin-free" casein hydrolyzed with trypsin supplies material that influences greatly the response of *L. leichmannii* (ATCC 4797) to vitamin $B_{12}$ (367) and this is true with respect to the response of other organisms to thymidine (228). This material also supplies substances whose effect on growth is augmented by reducing agents (367). Consequently, in media containing a tryptic digest of casein the possible influence of these phenomena on the validity of assays must be given recognition. With some of these microaerophilic organisms, *e.g.*, *L. lactis* (ATCC 8000), the effect of oxygen tension on microbial growth is very marked, in fact, excellent growth can be obtained anaerobically in the absence of vitamin $B_{12}$ or desoxyribosides. This circumstance can be of importance in cases where variations in the diameter of assay tubes, and thus in the depth of the medium, can influence significantly the tension of oxygen.

With the discovery of the presence of the CN-group in vitamin $B_{12}$, a quantitative chemical method was devised in which the amount of hydrogen cyanide to be obtained *under rigidly controlled conditions* from a solution containing cyano-cobalamin (about 2 μg.) under exposure to brilliant light is measured (31, 32, 32a, 32b); the method involves the quantitative isolation and specific determination of cyanide in minute traces (about 0.05 μg.).

It has been reported (348, 349) that in mice made anemic by treatment with phenylhydrazine, vitamin $B_{12}$ increases the rate of formation of erythrocytes, but not the hemoglobin concentration. Since a graded response was obtained when the dosage of vitamin $B_{12}$ was varied over the range of 1 to 4 μg. injected daily for 4 days, the development of an assay procedure was suggested. The remarkable feature of these observations is the apparent synthesis of red blood cells containing little or no hemoglobin the results need confirmation.

Many studies have been made of the vitamin $B_{12}$-requirement of animal species, particularly rats, pigs and chicks, and satisfactory biological assays for the vitamin have been devised using both the rat and the chick (100, 175). These investigations are an outgrowth, in part at least, of the long search for the "animal protein factor"; this particular aspect of the study of vitamin $B_{12}$ has been reviewed recently (399).

### *D. Relation of vitamin $B_{12}$ to "animal protein factor" (APF)*

It has been recognized since about 1932 (200) that rats and chicks require for their optimal nutrition something associated with animal protein and neither supplied by materials of vegetable origin nor associated with the amino acids of animal proteins. Even earlier studies by Coward, Key and Morgan (74) can be considered to form the basis of the subsequent developments. Deficiency of APF is evidenced not only by depression of growth, the severity of which may be great, but also by a markedly diminished hatchability of eggs and by the decreased viability of the offspring in both rats and chicks. Although ordinary crude casein supplies the factor, prolonged special extraction procedures can deprive it of a "Factor X" (57), so that the "vitamin-free" casein thereby obtained can be used with considerable success in lieu of a vegetable protein. The growth-promoting factor also is supplied by other materials, *e.g.*, meat scrap, "fish solubles," liver and liver extracts, and cow manure; however, yeast appears to be completely free of the substance, and, accordingly, in the early studies, large amounts of yeast were used to supply the other factors of the B-complex. Eventually it was found that the effect of the long sought for APF could be duplicated with crystalline vitamin $B_{12}$ (238). The Zuckers (399) have suggested that it may be somewhat inappropriate to include vitamin $B_{12}$ among the vitamins of the B-complex, since this generic term was applied originally to the water-soluble factors found in *yeast*. However, the functional rather than a saccharomycotic definition for the term "vitamin B-complex" seems more appropriate, as has been pointed out by Jukes (173). The review of the Zuckers (399) leaves no doubt concerning the difficulties and confusion attending the prolonged investigations of the APF and eventually culminated, in part at least, by the demonstration of the APF-action of cyano-cobalamin. Particularly prominent was the difficulty encountered in consistently obtaining a definite deficiency of APF in chicks and rats. Several factors contributed to this difficulty; among these were variations in the composition of the diets employed (*e.g.*, high protein intake favors the development of deficiency), the remarkable carry-over of vitamin $B_{12}$ from mother to off-spring, and the prolonged retention of the factor once it was "deposited" in the tissues. However, by feeding rats and chicks on deficient diets for two or more generations, severe deficiency states were obtained with reasonable consistency. Finally, the individual requirement for the vitamin was found to be so low that the difficulty of rigidly excluding it from diets may readily be appreciated; thus, young rats have been shown (113) to require only 0.1 $\mu$g. of vitamin $B_{12}$ when injected weekly, while, under the same conditions, chicks need about five times as much, or between 15 and 30 $\mu$g. per kilogram of diet (318). It will be obvious that on a weight-basis the requirement of these species of *growing* animals, with a high ratio of surface area to body weight, is enormously greater than is that of adult man; at least, as may be judged by the response of patients with pernicious anemia to the *parenterally* administered vitamin.

It is of considerable pharmacologic interest to note that the feeding of desiccated thyroid (or its equivalent, *e.g.*, suitably iodinated casein) to chicks, rats, or mice greatly favors the development of a deficiency of vitamin $B_{12}$ (257). This phenomenon may be regarded as due to the metabolic stimulation and resultant increase in nutritional requirements caused by thryoxine; due to this effect of the hormone, the rate of growth may be restricted by the need for dietary factors supplied in less than optimal amounts. This view is supported by other findings, for example, that of Ershoff (101), who showed that under certain circumstances the administration of thyroid substance at high levels leads to a marked decrease in survival that cannot be corrected by vitamin $B_{12}$, but is prevented by high dietary levels of whole liver and certain other materials.

The additional "APF-effect" of various antibiotics, when added to the diets deprive it of a "Factor X" (57), so that the "vitamin-free" casein thereby obtained can be used with considerable success in lieu of a vegetable protein. The growth-promoting factor also is supplied by other materials, *e.g.*, meat scrap, "fish solubles," liver and liver extracts, and cow manure; however, yeast appears to be completely free of the substance, and, accordingly, in the early studies, large amounts of yeast were used to supply the other factors of the B-complex. Eventually it was found that the effect of the long sought for APF could be duplicated with crystalline vitamin $B_{12}$ (238). The Zuckers (399) have suggested that it may be somewhat inappropriate to include vitamin $B_{12}$ among the vitamins of the B-complex, since this generic term was applied originally to the water-soluble factors found in *yeast*. However, the functional rather than a saccharomycotic definition for the term "vitamin B-complex" seems more appropriate, as has been pointed out by Jukes (173). The review of the Zuckers (399) leaves no doubt concerning the difficulties and confusion attending the prolonged investigations of the APF and eventually culminated, in part at least, by the demonstration of the APF-action of cyano-cobalamin. Particularly prominent was the difficulty encountered in consistently obtaining a definite deficiency of APF in chicks and rats. Several factors contributed to this difficulty; among these were variations in the composition of the diets employed (*e.g.*, high protein intake favors the development of deficiency), the remarkable carry-over of vitamin $B_{12}$ from mother to off-spring, and the prolonged retention of the factor once it was "deposited" in the tissues. However, by feeding rats and chicks on deficient diets for two or more generations, severe deficiency states were obtained with reasonable consistency. Finally, the individual requirement for the vitamin was found to be so low that the difficulty of rigidly excluding it from diets may readily be appreciated; thus, young rats have been shown (113) to require only 0.1 μg. of vitamin $B_{12}$ when injected weekly, while, under the same conditions, chicks need about five times as much, or between 15 and 30 μg. per kilogram of diet (318). It will be obvious that on a weight-basis the requirement of these species of *growing* animals, with a high ratio of surface area to body weight, is enormously greater than is that of adult man; at least, as may be judged by the response of patients with pernicious anemia to the *parenterally* administered vitamin.

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The additional "APF-effect" of various antibiotics, when added to the diets of growing animals of various species, *e.g.*, chicks, turkeys, rats, mice and swine, is worthy of comment. Since it has been reported (194) that the prolonged oral administration of aureomycin to patients with pernicious anemia in relapse causes a delayed but definite hematologic response, a consideration of the effect of antibiotics is quite in order.

It was first reported that products derived from the medium in which aureomycin is produced promote the growth of chicks (318) and swine (75, 177) and that this effect cannot be attributed to vitamin $B_{12}$. This observation has been confirmed widely and extended to other animal species, as has been indicated. The effect of the crude mixture of products derived from culturing species of *Aureomyces* also can be obtained with various highly purified antibiotics, singly or in admixture, *e.g.*, aureomycin, terramycin, streptomycin and penicillin. Responses also can be obtained with succinylsulfathiazole or 3-nitro-4-hydroxyphenyl-arsonic acid, but these compounds are less effective than repeatedly recrystallized aureomycin (316). It is of interest that apparent growth responses to both these compounds had been recorded earlier (25, 222, 368). There is evidence that antibiotics differ appreciably in their effectiveness in promoting growth in different species. In cattle and sheep, where bacterial action in the rumen is essential to the digestion of cellulose, antibiotics not only have failed to promote growth, but actually have markedly inhibited it (71, 197). However, recent work, as yet unpublished, indicates that even in ruminants low levels of aureomycin may improve the rate of growth (171).

The first phase of the mechanism of action of the antibiotic compounds almost certainly is to be attributed to the inhibition of certain intestinal microorganisms, but whether the end-result is attained by the prevention of the formation of compounds inhibitory to growth, by the prevention of the destruction of growth-promoting compounds, or by increasing the synthesis of such stimulatory substances, as a result of marked changes in the flora of the intestinal tract, or by some other means, has not been elucidated.

With respect to the hematologic responses to aureomycin noted by Lichtman, Ginsberg and Watson (194), which were obtained either with or without the concurrent administration of vitamin $B_{12}$, the inference might be drawn that aureomycin promotes the formation of vitamin $B_{12}$, its more efficient utilization, or both. However, as was considered by these workers, the hematologic effects of aureomycin could more logically be attributed to an increased bacterial production of readily utilized derivatives of folic acid.

Very pertinent to these findings in man are those of Waisman and associates in rats (353). Although aureomycin was without influence on the growth of rats made deficient in folic acid by feeding a "purified" diet containing a sulfonamide, the orally administered antibiotic overcame the toxicity of a potent antagonist of folic acid ('Aminopterin,' *v.i.*). These findings strongly indicate that aureomycin in some manner promotes either the formation or the utilization, or both, of the citrovorum factor (*v.i.*), a derivative of folic acid which, unlike the parent compound, is able effectively to reverse the toxic effects of 'Aminopterin' (41, 46, 47, 230, 274). According to this view, the sulfonamide, of growing animals of various species, *e.g.*, chicks, turkeys, rats, mice and swine, is worthy of comment. Since it has been reported (194) that the prolonged oral administration of aureomycin to patients with pernicious anemia in relapse causes a delayed but definite hematologic response, a consideration of the effect of antibiotics is quite in order.

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Very recently it was reported by Foy *et al.* (106) that the daily *parenteral* administration of crystalline penicillin G (400,000 units daily) induced an excellent hematologic response in a patient with the megaloblastic anemia of pregnancy. Since this condition rarely responds to vitamin $B_{12}$ but is very susceptible to therapy with folic acid (*v.i.*), the hypothesis presented above appears to be applicable in this situation as well.

### *E. Clinical efficacy*

About three years have elapsed since the cobalamins became available for clinical testing and therapeutic use. During this period crystalline cyano-cobalamin, and concentrates containing mixtures of hydroxo-, cyano-, and possibly other cobalamins, have been shown to be very effective in correcting both the hematologic and neural lesions in pernicious anemia patients (132, 135, 169, 195, 225, 273, 369, 370). Definite but less striking results have been obtained in the treatment of the clinical and hematologic manifestations of tropical sprue (166, 302, 303, 340). However, the megaloblastic anemia occasionally associated with pregnancy, and that sometimes seen in infancy, respond but rarely to vitamin $B_{12}$, although both conditions are highly susceptible to therapy with folic acid (23, 88, 118, 198, 342).

Results obtained in acute experiments on pernicious anemia patients in relapse have indicated that the daily parenteral administration of 1 μg. of vitamin $B_{12}$ will induce maximal hematologic responses in most cases (133, 225). It has been suggested tentatively, therefore, that approximately 1 μg. of crystalline cyano-cobalamin has the clinical activity of 1 U.S.P. parenteral unit of liver extract. Some data have accumulated (169) to indicate that amounts larger than 1 μg. daily may give more nearly optimal results, but it seems likely at this time that the minimal daily requirement for the proper treatment of pernicious anemia does not exceed 3 μg. per day. Administration of larger amounts of vitamin $B_{12}$ are seldom necessary, but it is conceivable that an occasional patient might have a greater requirement. In actual practice, patients in relapse are often treated with daily intramuscular doses of 15 μg. or more. The various forms of cobalamin so far tested are fully effective in the treatment of pernicious anemia (67, 167, 195, 251, 273, 345), but data are insufficient to permit a statement concerning the relative clinical potencies of the various forms.

While the original material was isolated from liver, it was discovered shortly afterwards that vitamin $B_{12}$ is produced by many microorganisms. The first report of the clinical efficacy of material derived from a bacterial source, an unidentified organism found in hen feces, appeared in 1948 (320). At the present time, the cobalamins are being produced commercially from various bacterial sources, in particular through the use of one or another species of *Streptomyces*. More than one derivative of cobalamin may be present in such products; furthermore, variation in the strain or species, and in the conditions of growth, may alter the total amount, as well as the form, of the vitamin produced. For clinical use the cobalamins are frequently provided in the form of so-called "concentrates," in which the derivatives of cobalamin may comprise only about 1 per cent of the solid material present. However, crystalline cyano-cobalamin isolated from bacterially produced material also is available commercially.

The question naturally arises as to whether all of the anti-pernicious anemia activity of liver extracts is the result solely of the cobalamins present or whether other substances present in liver extracts may of themselves, or in conjunction with cobalamin, have clinical activity. The writers believe there is no convincing evidence that present-day refined liver extracts contain clinically significant amounts of anti-pernicious anemia substances other than the various forms of cobalamin, and it now appears that these pure compounds, in sufficient dosage, are fully as effective in the management of pernicious anemia as are any of the various extracts of liver.

The findings reviewed in the foregoing paragraphs justify the conclusion that cyano-cobalamin and certain of its derivatives are the constituents of liver extracts responsible for their efficacy in the treatment of pernicious anemia. When a vitamin is effective in the management of a disease state, it follows logically that the disease state is the result of an inadequate concentration of the vitamin, or metabolically active derivatives of it, in the tissues. Accordingly, the syndrome, pernicious anemia, must be attributed to a deficiency of vitamin $B_{12}$.

### *F. Etiology of pernicious anemia*

1. *Concept of "extrinsic factor" and "intrinsic factor."* The fundamental clinical investigations which permit at least a partial explanation of the mechanism of this deficiency were conducted by Castle and his associates (59) over twenty years ago. These studies showed that the oral administration of normal gastric juice together with beef muscle to patients with pernicious anemia evoked an hematologic response, although neither material was effective by itself. Thus, it was demonstrated that the gastric secretion of patients with pernicious anemia is deficient in a substance present in normal gastric juice which is essential for normal hematopoiesis. This heat-labile substance, which is found in normal human gastric juice, and in the gastric and intestinal mucosa and contents of other species, particularly swine (276, 325), was termed by Castle, the *intrinsic factor*. The unknown heat-stable substance in meat and certain other substances was called the *extrinsic factor*. Although, at first, various techniques of incubation of sources of extrinsic factor with intrinsic factor were employed, it was found

that an hematologic response occurred even though the two components were administered separately, provided that the interval between the two dosages did not exceed six hours (61).

During the following years much work was performed in an effort to extend the knowledge of these two factors. Thus, evidence was presented that in addition to meat, such foodstuffs as eggs, rice polishings, wheat germ, autolyzed yeast, milk, crude casein, and certain other animal proteins (218) apparently possess extrinsic factor activity. Castle postulated that the intrinsic factor interacted in some manner with the extrinsic factor, probably in the small intestine, and that the product of this interaction was stored in and recoverable from the liver. It was clearly stated, however, that the interaction did not necessarily imply a chemical alteration of either the intrinsic or the extrinsic factor (62). It is important to note in this connection that incubated mixtures of normal human gastric juice (intrinsic factor) and beef muscle (extrinsic factor), like intrinsic factor itself, are rendered ineffective hematopoietically by heating to 70° to 80°C for 30 minutes (62). This indicates that a new thermostable product of the interaction of the two factors is not formed during the incubation period, as had been claimed by Klein and Wilkinson (186). It has been shown that a further addition of unheated gastric juice to a heat-inactivated mixture of intrinsic and extrinsic factors restored oral activity (61). This observation presumably signifies only that the heating had inactivated the intrinsic factor component and that the oral activity of extrinsic factor is dependent on the presence of undenatured intrinsic factor.

It was found (276, 325) that the dried and powdered defatted wall of the stomach of the hog had oral activity in pernicious anemia; however, this activity could be abolished by heating. It was evident, therefore, that this dried gastric tissue supplied both the heat-stable extrinsic factor and the heat-labile material also found in gastric juice. Commercial application of this observation is found in Powdered Stomach, U.S.P. (*e.g.*, 'Ventriculin'), an effective daily oral dose of which is about 40 grams. It was found also that the activity of orally administered crude liver extracts is augmented by the simultaneous administration of normal gastric juice or gastric mucosa (103, 252). Clinical application of this finding is made through the use of commercially available mixtures of liver and stomach for the oral treatment of pernicious anemia. By definition then, it would seem that liver extracts contain extrinsic factor, in addition to the anti-pernicious anemia substance now identified as vitamin $B_{12}$.

More recently it was demonstrated that the *oral* activity of liver extracts further concentrated by adsorption on and elution from charcoal, and intended only for intramuscular administration, also can be augmented by the simultaneous administration of normal human gastric juice (19). Daily oral dosage with one ml. (15 U.S.P. units) of this material was shown to produce little or no hematopoietic effect, but administration together with normal gastric juice caused an unequivocal hematologic response. Accordingly, it would appear that liver extracts purified in this manner contain, as well as the parenterally active anti-pernicious anemia substance, a material that behaves, by definition, as the extrinsic factor.

2. *Demonstration of extrinsic factor activity of cyano-cobalamin.* The possibility that the two substances, extrinsic factor and the anti-pernicious anemia factor, were identical was appreciated, of course, although it had been reported (218) that the anti-pernicious anemia activity of liver extracts could be destroyed by treatment with sulfuric acid without loss of extrinsic factor activity. As soon as crystalline vitamin $B_{12}$ (cyano-cobalamin) became available, a test of its oral activity in the absence and in the presence of intrinsic factor was made by Boston and Cleveland groups working collaboratively (19). It was found that the daily oral administration of relatively small amounts of cyano-cobalamin (5 or 10 $\mu$g.) usually had little or no hematologic effect in patients with pernicious anemia in relapse, but when these were administered simultaneously with normal human gastric juice, a definite response occurred. On the other hand, when each of the two materials was administered daily, *but with dosage separated by an interval of twelve hours*, no hematologic effect was noted. These observations indicated that a constituent of gastric juice is involved in the utilization of orally administered vitamin $B_{12}$. Since, in these experiments, vitamin $B_{12}$ behaved in all respects as the extrinsic factor, and since the cobalamins are responsible for the anti-pernicious anemia activity of "purified" liver extracts, it followed that the two activities could be attributed to the same substance, namely, vitamin $B_{12}$ (and related cobalamins). It could be concluded, further, that the pernicious anemia patient, because of a deficiency in gastric secretion, fails properly to absorb, or in some other manner to utilize, the cobalamins, as a consequence of which a deficiency of vitamin $B_{12}$ develops. The results which form the basis of these conclusions have been confirmed extensively (21, 50, 51, 130, 131, 136, 301, 303, 344a).

Other evidence indicates that the utilization of vitamin $B_{12}$ following oral administration may be far from complete, even in the presence of presumably adequate amounts of intrinsic factor. Thus, a patient with an initial erythrocyte count of 2,160,000 per cmm. of blood, who showed no reticulocyte response when vitamin $B_{12}$ (5 $\mu$g.) was administered daily by mouth (with gastric juice, 125 ml., *given twelve hours subsequently*), and only a small response (3.8 per cent reticulocytes) when the two materials were given simultaneously, developed a much greater reticulocytosis (14 per cent) when the same dose of vitamin $B_{12}$ was given daily by intramuscular injection during a third period (19). More recently, Castle (58) has obtained further evidence that the proportion of the oral dose which is utilized as a result of the administration of intrinsic factor may be quite small. Thus, in a patient who had shown no hematologic response to a daily oral dose of 1 $\mu$g. of vitamin $B_{12}$ (period 1), but a definite response to the same dose when given together with 10 ml. of gastric juice daily (period 2), a second reticulocyte response followed the administration of 5 $\mu$g. of vitamin $B_{12}$ and 10 ml. of gastric juice daily (period 3). An additional response was obtained when a daily dose of only 1 $\mu$g. of vitamin $B_{12}$ was given *parenterally* during a final period. These findings presumably indicate that, in this patient at least, considerably less than 20 per cent of the daily oral dose of 5 $\mu$g. of vitamin $B_{12}$ may be utilized as a result of the co-administration of only 10 ml. of normal human gastric juice.

Of considerable interest is the conclusion of von Bonsdorff (351) that the megaloblastic anemia which often appears in individuals carrying the fish tapeworm, *Diphyllobothrium latum*, is due to an interference in the reaction between intrinsic and extrinsic factors, since administration of normal human gastric juice and sources of extrinsic factor caused no hematologic response until after the worm was expelled. Conceivably, the worm, lying in the upper part of the small intestine, inactivates either vitamin $B_{12}$ or the intrinsic factor, but the latter possibility is considered much the more reasonable.

That the cobalamins may more properly be regarded as extrinsic factor, than as anti-pernicious anemia factor, is suggested by recently published findings. Thus, in tissue culture experiments using the bone marrow of patients with pernicious anemia in relapse, Lajtha (189, 190) found that cyano-cobalamin caused no maturation of megaloblasts, whereas disappearance of the megaloblasts was induced by minute amounts of either folic acid or citrovorum factor (leucovorin; folinic acid). Surprisingly enough, however, the simultaneous addition of both normal gastric juice (which by itself was inactive) and extremely small amounts of cyano-cobalamin also caused a prompt disappearance of the megaloblastic cells (190). As a result of these observations, the authors have suggested that the ultimate anti-pernicious anemia factor is a complex of cobalamin and intrinsic factor, which functions by preventing or removing an inhibition of the action of a compound derived from folic acid. It was postulated that since the cobalamins evoke an hematopoietic response upon intramuscular administration to patients, *an extra-gastric intrinsic factor must exist.*

Using a quite different technique, Horrigan and his coworkers have reported (160) that injection of a single small dose of cyano-cobalamin (1 μg. in 1 ml.), into a marrow cavity (from which 2 or 3 ml. of marrow and blood had been aspirated) of patients with pernicious anemia in relapse, causes local maturation of megaloblasts *in situ*, but not at points distant from the area of injection. However, neither folic acid nor citrovorum factor was active in this type of experiment. Although the findings of the British and the American workers appear somewhat difficult to reconcile, it is suggested that the failure of the folic acid-type of compounds may be due to their rapid removal from the site of administration in the marrow. On the other hand, the apparent tendency of vitamin $B_{12}$ to be "bound" by certain proteins (28) may serve to prevent its entrance into the circulation, so that a local effect could be exerted in a manner resembling that observed by Lajtha *in vitro* (190).

### G. *Intrinsic factor*

Although the intrinsic factor has not yet been isolated in pure form and perhaps only a little more is known concerning its function than was recorded in 1929 by Castle, considerable progress in our understanding of its chemical nature and biological role may be anticipated in the near future. Early studies showed that its activity is destroyed by boiling for 5 minutes or by heating to 70°–80° C. for 30 minutes. Also it was shown that the factor will pass a Berkefeld V filter, is non-dialyzable, and is not identical with pepsin, rennin or trypsin (59).

The intrinsic factor is secreted by the stomach of normal man and various mammalian species. In swine, it is probably produced principally by the glands in the pyloric end of the stomach and, perhaps, by Brunner's glands of the duodenum (209). In man, however, there is evidence to suggest that it is secreted principally in the fundic portion of the stomach (105).

It should be pointed out that the deficiency of intrinsic factor, which characterizes the pernicious anemic state, is not necessarily an absolute one. It was shown in 1937 that the pooled (scanty) secretions of the stomach of groups of patients with pernicious anemia in relapse were able to induce a response in other patients, when administered simultaneously with beef muscle (123). The occasional hematologic response of patients with pernicious anemia given oral doses of crystalline cyano-cobalamin in doses of 5 or 10 μg. daily without the co-administration of intrinsic factor (21, 143), presumably is to be accounted for by the secretion of some residual intrinsic factor by a gastric mucosa the function of which, in this respect, has not been lost entirely. The fact that such a response to vitamin $B_{12}$ can occur makes imperative the daily administration of any source of extrinsic factor during a period of at least ten days prior to testing the additional effect of a material with possible activity as intrinsic factor. Neglect of this basic tenet of biologic assay appears to have been responsible for the erroneous conclusions drawn by a number of investigators.

1. *Aminopolypeptidase.* On the basis of experiments designed to concentrate the aminopolypeptidase activity of hog gastric tissue, wherein there also was obtained a concentration of intrinsic factor activity, Ågren and Waldenström postulated the possible identity of the two materials (1, 2). However, recent collaborative experiments by workers in Boston, New Haven and Cleveland (272) showed not only that calf thymus aminopolypeptidase is devoid of intrinsic factor activity, but also that normal human gastric juice, unlike the gastric tissue of swine, has no demonstrable aminopolypeptidase activity. It seems likely, therefore, that aminopolypeptidase is not intrinsic factor and that the intrinsic factor activity of concentrates of gastric aminopolypeptidase was the result of the concentration of two heat-labile proteins with somewhat similar physico-chemical properties, or of a complex of the two proteins.

2. *Lysozyme.* This enzyme was reported by C. E. Meyer and associates (210) to have activity as intrinsic factor. Concentrates of egg white, high in lysozyme activity, produced rather inconclusive results in clinical studies which unfortunately were interpreted positively by Meyer. That lysozyme is not identical with the intrinsic factor also is indicated by the observation that gastric lysozyme is produced by the surface epithelial, rather than the glandular, gastric cells; further, the enzyme is present in greater amounts in the gastric secretions of pernicious anemia patients than in normal individuals (126). Recently, Hall (130) has demonstrated that a complex of egg lysozyme and vitamin $B_{12}$ has no greater activity than an equivalent amount of the free vitamin when administered orally to patients with pernicious anemia in relapse.

3. *Relation of folic acid to intrinsic factor.* A claim that "folic acid acts like 'intrinsic factor' since it promotes utilization of orally administered vitamin $B_{12}$ (extrinsic factor?) in subminimal doses," has been made by Leo Meyer

and associates (212). This hypothesis was based on the conclusion that patients given simultaneously both vitamin $B_{12}$ and folic acid by mouth, each in a supposedly suboptimal amount, developed reticulocyte responses and a rate of erythrocyte regeneration greater than was to be expected on theoretical grounds. No direct evidence was obtained to show that the absorption or utilization of vitamin $B_{12}$ was altered. Interpretation of such an experiment, where two substances are given simultaneously, each potentially capable of evoking a response, is hazardous indeed. The marked individual variations in the hematologic responses of patients with pernicious anemia, so frequently encountered by workers in this field, makes it quite impossible accurately to predict the extent of the hematopoietic response to be anticipated in any individual patient. Although tables have been constructed (165) in which the theoretically maximal reticulocyte response, to be expected following optimal hematopoietic stimulation, is related to the initial erythrocyte count, it is frequently forgotten that these tables are based on "the means of a whole series of observations. Obviously, many responses will fall above and many below the 'expected' or standard curves so derived. Investigators have not always recognized this fact in the effort to generalize conclusions regarding dosage level or activity from a single case or a very few cases. It is probable that many of the discrepancies in the chemical fractionation of liver have arisen from interpretation of differences between individual patients rather than true differences in the activities of fractions tested" (Darby, 80).

In further consideration of the claim of Meyer it may be mentioned that the physico-chemical properties of folic acid are quite different from those exhibited by intrinsic factor, which behaves like a protein; also, there is no evidence to suggest that folic acid increases the secretion of intrinsic factor by the gastric mucosa of patients with pernicious anemia. In fact, it is generally recognized that the gastric atrophy and achylia characteristic of that disease are not ameliorated by treatment with folic acid. It must be concluded that the evidence in support of the concept that folic acid either is the intrinsic factor, or exerts intrinsic factor-like activity, is wholly inadequate.

4. *Vitamin $B_{12}$-binding and intrinsic factor.* Ternberg and Eakin (332), without presenting any supporting clinical evidence, have suggested that a "vitamin $B_{12}$-binding" protein termed *apoerythein* which they find in normal gastric juice and gastric tissues is intrinsic factor. The gastric juice of patients with pernicious anemia appeared to contain reduced amounts of the substance, but this point is of doubtful significance since the gastric achylia of pernicious anemia leads to a great reduction in the amount of most substances normally secreted by the gastric mucosa.

The "binding" phenomenon was measured by observing the effect of the *unheated* protein in preventing the utilization of vitamin $B_{12}$ by microorganisms which require the vitamin for growth (for this purpose a strain of *E. coli* was used which, appropriately inhibited by a sulfonamide, responds to vitamin $B_{12}$). It was suggested that the "binding-protein" combines with vitamin $B_{12}$ to form a complex which they have named *erythein*. Although several substances which bind the vitamin were found in gastric tissue, only one was thought to occur in gastric juice.

It is quite evident that many substances are capable of "binding" vitamin $B_{12}$, and with varying degrees of intensity (28, 249), so that it would appear unsafe, without good clinical evidence, to conclude that any binding-phenomenon is to be attributed to intrinsic factor. Since none of the concentrates of the Texas group has been reported to have been tested in patients with pernicious anemia, the only way in which intrinsic factor activity can be *proved* to be present, the claims of these workers are impossible to evaluate.

It is entirely possible that intrinsic factor, when obtained in a state of chemical purity, will prove capable of forming a complex with various cobalamins, and possibly to exert its physiologic function in this manner. However, the lack of specificity of this "binding" suggests that microbial assays for materials with apoerythein activity may be of dubious value as a means of guiding the concentration of the intrinsic factor.

Beerstecher (14, 15) recently has claimed that intrinsic factor actually is derived from saliva. This claim is based on his finding that saliva is a potent source of material that binds vitamin $B_{12}$, making it unavailable for microbial utilization. This worker appears to accept without question the unproved hypothesis of Ternberg and Eakin (332) concerning the relationship of apoerythein to the intrinsic factor. He postulates that in the presence of achlorhydria the intrinsic factor of saliva is inactivated. Although achlorhydria is a constant concomitant of pernicious anemia, it is present, of course, in other conditions and in many apparently healthy persons. Beerstecher suggests that hydrochloric acid is needed to prevent the inactivation of the intrinsic factor of saliva and thus, apparently, he would consider pernicious anemia to be a resultant of achlorhydria. Again, the only established test for intrinsic factor activity, *i.e.*, the suitable oral administration (with extrinsic factor) to a patient with pernicious anemia in relapse, was not performed. Many experiments carried out during the past twenty years, and entirely disregarded by Beerstecher, appear to invalidate these claims completely. Castle and his associates have shown (63) that human saliva is inert when given with beef muscle at pH 5, while gastric juice is active under these conditions. Mixtures of beef muscle and either gastric juice or fresh hog stomach, incubated *in vitro* after acidification to pH 2.5 to 3.5, are inert hematologically when given orally to pernicious anemic subjects, unless the pH is restored to a level higher than 3.5 (usually 5 or 7) (61). Normal human gastric juice, administered orally at pH 7 with vitamin $B_{12}$, is active even in amounts as small as 10 ml., as has been mentioned previously. The simultaneous administration of beef muscle, pepsin, and hydrochloric acid is ineffective in pernicious anemia patients who may reasonably be presumed to have been swallowing normal amounts of their own saliva (63). Finally, if Beerstecher should prove to be correct in his claim that the vitamin $B_{12}$-binding activity of gastric juice actually is due to the admixture with it of swallowed saliva, the known clinical inactivity of saliva as a source of intrinsic factor would contribute further to the doubt concerning the correctness of the conclusion of Ternberg and Eakin that the vitamin $B_{12}$-binding substance of gastric juice is the intrinsic factor.

5. *Gastric mucoprotein.* Glass and associates (121) believe that they have been able to identify intrinsic factor with *gastric mucoprotein* derived from human sources. Although the fractions studied were stated to consist primarily of mucoprotein, and electrophoretic studies were believed to indicate the presence of a single substance, it will be appreciated that electrophoresis, by itself, is not necessarily a reliable indicator of homogeneity (155). When the mucoprotein preparations were administered in daily oral doses of 50 to 100 mg. to patients with pernicious anemia in relapse, together with 20 to 30 μg. of vitamin $B_{12}$, definite hematologic responses were obtained. However, the possibility that intrinsic factor actually was associated with the mucoprotein, and that the two substances are not identical, cannot be excluded. As will be pointed out, the authors of this review and their colleagues, in collaboration with Castle and his associates, have obtained good hematologic responses to relatively crude fractions of human gastric juice, administered concurrently with cyano-cobalamin (5 μg. daily), in which the effective oral dose of the fraction of gastric juice was only 0.6 mg. daily. Further investigation soon should clarify the question of the role of mucoprotein.

6. *Concentration of intrinsic factor.* Investigations in the authors' laboratories (Prusoff and associates, 249) have resulted in considerable concentration of the intrinsic factor activity of powdered stomach ('Ventriculin,' Parke, Davis and Company). After extraction of the activity with a two per cent solution of sodium chloride, adjustment of the extract to pH 9 (to inactivate pepsin), followed by the removal of a large amount of inert material at pH 1.5 (including much of the mucoprotein), fractional precipitation with ammonium sulfate was carried out at pH 4.5. In the following outline, the quantities referred to parenthetically, each derived from approximately *50 grams* of powdered stomach, were administered daily. Fraction B (*about 15 mg.*), precipitable by 55 per cent saturation with ammonium sulfate, after previous removal of fraction A (*about 210 mg.*) which was precipitated at 35 per cent saturation, had more activity than either fraction A, or fraction C (*about 570 mg.*), which was precipitated at full saturation after the removal of fractions A and B. Both fractions A and C contained some activity, although considerably less than fraction B which contained a very much smaller amount of total solids than either of the other fractions. Possibly, small amounts of mucoprotein present in the fractions of gastric tissue, despite the previous precipitation at pH 1.5, influenced the "sharpness" of the precipitations with ammonium sulfate and prevented better separation of the intrinsic factor from inert materials. Investigation of these fractionation procedures is being continued.

Preliminary experiments with normal human gastric juice, held for varying periods in the frozen state, also have been carried out by the authors and their associates, in collaboration with Castle and his group in Boston. A previous report (149) that intrinsic factor activity can be precipitated from gastric juice by half-saturation with ammonium sulfate has been confirmed and extended.

After holding the gastric juice at pH 9 for at least 30 minutes to inactivate pepsin, and adjustment to pH 4.5, saturation with ammonium sulfate was carried out and the precipitate thereby obtained was freed of the sulfate ion by dialysis. The aqueous solution of the precipitated material was adjusted to pH 1.5 and insoluble material was removed (as was the case with extracts of powdered stomach tissue), while the supernatant fraction, after adjustment to pH 4.5, was half-saturated with ammonium sulfate. In one instance the precipitate obtained in this manner (0.6 mg. per 100 ml. of original gastric juice) was much more active as intrinsic factor than was the material precipitated subsequently (5 mg. per 100 ml. of original gastric juice) by raising the ammonium sulfate concentration to the level of saturation. Further studies on these and other fractions are in progress.

Since all assays of intrinsic factor activity are carried out in patients with pernicious anemia in relapse previously proved to be unable effectively to utilize orally administered cyano-cobalamin, the rate of progress to be anticipated in this research program inevitably is slow. However, the conclusions drawn are based on the only assay of proved validity. Concurrent studies are being made of the various fractions as sources of vitamin $B_{12}$-binding materials, and, more recently, for their influence on the "ripening" of megaloblasts in tissue culture preparations of pernicious anemic bone marrow (in the presence of vitamin $B_{12}$), as described by Lajtha (189, 190). With respect to the "binding" of vitamin $B_{12}$, the experience in our laboratories has been (249) that fractions of powdered stomach having the highest activity in the human assays do not have, per mg. of protein, the highest capacity to "bind" cyano-cobalamin. Also, Hall (130) has reported that two extracts of the gastric mucosa of swine, which showed a strong binding capacity for vitamin $B_{12}$, possessed little or no intrinsic factor activity when tested clinically.

7. *Mechanism of action.* Much speculation has been engendered by the finding that for the utilization of even very minute amounts of orally ingested vitamin $B_{12}$, a heat-labile material, intrinsic factor, is essential. Chow has shown (65) that oral administration of vitamin $B_{12}$ over a period of several months or in a single massive dose (5.0 mg. per adult) did not result in the appearance of vitamin $B_{12}$ activity in the urine. A major portion of the ingested vitamin appeared in the feces. When the vitamin was given intravenously to man, the blood level decreased rapidly and much of the administered vitamin could be recovered in the urine.

In another study, Chow *et al.* (66), using radioactive cyano-cobalamin (obtained by growing vitamin $B_{12}$-producing organisms in the presence of cobalt-60) (64), found that only a small fraction of the radioactivity appeared in the urine following the oral administration of relatively massive doses of the labeled vitamin to normal rats.

Determination of the effect of intrinsic factor on the utilization of orally administered vitamin $B_{12}$, by comparison of the amounts of vitamin excreted in the feces during periods of the daily administration of fixed amounts of cyano-cobalamin, is a difficult procedure. This is because relatively large amounts of vitamin $B_{12}$-like substances are found in the fecal excretions of patients with pernicious anemia, as well as in those of normal human subjects. The substances excreted not only are active microbially but are active hematopoietically (23, 49, 120). Callender and Spray (49), using extraction procedures that almost certainly were incomplete, found variable amounts of microbially active materials corresponding to a daily excretion of 19, 3.8 and 22 μg. of vitamin $B_{12}$, respectively, by three patients with untreated pernicious anemia; the corresponding figure for a normal subject was 8 μg. It is logical to assume that this fecal excretion of materials which behave like vitamin $B_{12}$ reflects the synthetic activity of microorganisms in areas of the intestine where the vitamin is not absorbed or is due to the unavailability of the vitamin for absorption because of a "binding" phenomenon. That vitamin $B_{12}$ is "bound" to a marked degree by such organisms as *E. coli* has been shown (87). These findings indicate clearly that ordinary measurements of the fecal excretion of vitamin $B_{12}$ before and during the administration of sources of the intrinsic factor will give little or no indication of the amount of the vitamin which may be removed from the intestinal tract as the result of an influence of this heat-labile factor.

Studies are now in progress in our laboratories in which intensely radioactive crystalline cyano-cobalamin is being used to determine whether, in the absence of an adequate amount of intrinsic factor, even minute doses of vitamin $B_{12}$ fail to be absorbed and are excreted in the feces. If this should prove to be the case, it might be possible to assay various fractions for intrinsic factor in patients with pernicious anemia *in hematologic remission*. According to this concept, activity as intrinsic factor would be evidenced by a diminution in the proportion of a fixed small dose (1 to 5 μg.) of cyano-cobalamin-$Co^{60}$ excreted in the feces of patients given the vitamin together with a source of intrinsic factor, as compared with the amount excreted in the absence of intrinsic factor. Unfortunately, urinary excretion of the vitamin following oral administration is not of significant magnitude to permit absorption to be followed in this more simple manner (65, 66).

Although there appear to be no decisive experiments that bear precisely on the mechanism of action of intrinsic factor in facilitating the utilization of vitamin $B_{12}$ from the gastro-intestinal tract, speculation would suggest that intrinsic factor is concerned in some manner with the passage of the vitamin through membranes of certain cells (*e.g.*, cells of the intestinal mucosa) (272). On the other hand, there are as yet no data which exclude completely the possibility that the intrinsic factor functions in preventing the inactivation of the vitamin, or its "binding" by intestinal microorganisms, or through other less obvious mechanisms.

Pertinent to the concept of transport across cell membranes are the recent studies of Lajtha (189, 190), which have been mentioned previously. These studies, if confirmed, strongly indicate that an intrinsic factor also is critically involved in the utilization of vitamin $B_{12}$ by pernicious anemic bone marrow cells in tissue culture. It was observed that maturation of megaloblasts *in vitro* was not influenced by crystalline vitamin $B_{12}$ unless a small amount of sterilefiltered gastric juice was present. The investigators have suggested that the factor of gastric juice forms an essential complex with vitamin $B_{12}$ and that this complex is concerned with preventing the action of an inhibitor of cofactors derived from folic acid. Wolf and his co-workers (389) found no evidence for a chemical reaction between crystalline vitamin $B_{12}$ and normal gastric juice, although the techniques employed did not exclude the possibility that such a reaction may occur. The observations of Lajtha offer possibilities that are of particular pertinence to this portion of the review; for example, (1) a somewhat simpler system might be afforded for more fundamental studies of the mechanism of action of intrinsic factor; (2) a bioassay procedure might be made available, which, although requiring the marrow of patients with pernicious anemia in relapse and by no means simple in execution, would offer many advantages over the present difficult, cumbersome and time-consuming assays carried out exclusively *in vivo* in patients; and (3) possibly, even for the effective utilization of *parenterally* administered vitamin $B_{12}$, intrinsic factor may be essential; in this case, there would appear to be involved a hitherto unsuspected extra-gastric intrinsic factor, presumably transported by the blood, and found in adequate amounts in patients with pernicious anemia.

8. *Relation of intrinsic factor to the utilization of substances other than the cobalamins.* Intrinsic factor possibly may be involved in the utilization of an hematopoietic agent of an unknown nature, found particularly in extracts of autolyzed brewers' yeast (*Saccharomyces cerevisiae*). This statement is based on the observation of Strauss and Castle in 1932 (321) that the oral activity of 'Vegex' was augmented by the simultaneous administration of gastric juice, as was that of an alcoholic extract of autolyzed yeast, and on that of Ungley (341, 346), who noted a similar effect of gastric juice on the response to 'Marmite.' Various workers, using either animal or microbial assays, have been unable to find evidence of the presence of a significant amount of vitamin $B_{12}$ in brewers' yeast (399), of which 'Marmite' is an extract. Kodicek and Carpenter (187) found by rat assay that 'Marmite' contains approximately 60 μg. of pteroylglutamic acid per gram. The situation is complicated further by a finding of Ungley, who states (344), "In a patient with pernicious anemia of pregnancy an alcoholic extract of yeast was effective in doses which contained less than 40 μg. of folic acid tested both microbiologically and in rats for conjugates. Moreover, the daily excretion of folic acid in the urine during the period of administration of yeast was extremely low—only 1 to 5 μg. per day. There was no secondary reticulocyte response after the subsequent administration of 2.5 mg. of folic acid daily, although the average daily excretion of folic acid in the urine rose to 700 μg. per day. The evidence is against folic acid being the cause of the haematopoietic response observed with yeast."

Since the megaloblastic anemia of pregnancy characteristically fails to respond even to parenterally administered vitamin $B_{12}$, while folic acid ordinarily induces excellent hematological responses, this finding of Ungley seems to indicate that the yeast extract may have contained a substance resembling folic acid. However, the failure to find evidence of the presence of such a substance through the assays conducted in rats, and the failure of the patient to show an augmented urinary excretion of folic acid during the period of dosage, give no support to such a conclusion. Conceivably, the active substance in the yeast extract is formed physiologically as a result of *one* of the catalytic functions of folic acid. Thus, a compound could exist which, though able to promote hematopoiesis in a human subject, would not enable the profoundly folic acid-deficient rat to dispense with its multiple requirements for a utilizable form of pteroylglutamic acid. Such an hypothesis would suggest the possibility that the substance might be thymidine or might be involved in the synthesis of thymidine (see sections on folic acid and on mechanism of action).

Obviously, a statement as to the true significance of these observations with yeast extracts cannot be made at this time. That the results cannot be attributed to a form of vitamin $B_{12}$, in view of the fact that brewers' yeast is devoid of animal protein factor activity, appears to be a reasonable conclusion. It is equally difficult to attribute the results to conjugated forms of folic acid, in view of the results of Ungley (344). However, brief comment might be made concerning an unconfirmed report (48) which claimed that the conjugated form of folic acid, as found in yeast, was altered by incubation with gastric juice (*at pH 2*), although, in confirmation of our findings (361, 362), free folic acid was not released. Presumably it was suggested (48) that the altered conjugate of folic acid can be utilized only after further modification by other enzymes which, however, are unable to react with conjugate which has not been altered previously by the gastric enzyme. In support of these views the workers present only limited data which are not easily interpreted. Whether they offer any clue to the extrinsic factor activity of yeast extracts cannot be decided on the basis of the information now available. More intensive studies of the apparent extrinsic factor activity of a component of brewers' yeast would appear to be in order.

### H. *Vitamin $B_{12}$ in body fluids and in certain constituents of the diet*

Using *Euglena gracilis*, which requires vitamin $B_{12}$ for growth, Ross (260) observed an effect with amounts of the vitamin as small as 1 μμg. (*i.e.*, 0.000000001 mg.) per ml., but the method is of practical value only with amounts of 10 μμg. or more per ml. Depending on the degree to which normal sera were heated, the apparent content of vitamin $B_{12}$ was found to vary from an average value (40 subjects) of 185 μμg. (range: 20–350 μμg.) per ml., when heated at 70° for one-half hour, to 490 μμg. (range of twelve samples: 350–750 μμg.) per ml., when heated at 100° for one-half to one hour. Heating the samples at 100° at pH 10 for one-half hour, as would be expected (159a, 367), destroyed most of the activity. Cerebrospinal fluid analyses gave values from nil to 16 μμg. per ml., while urine appeared to contain from nil to 200 μμg. per ml. It is of considerable interest that sera, heated at 70°, from four cases of untreated pernicious anemia patients, did not stimulate growth of the organisms; even after heating at 100°, the activity obtained was much less than with the normal sera. When vitamin $B_{12}$ was given intramuscularly to two patients with pernicious anemia, stance through the assays conducted in rats, and the failure of the patient to show an augmented urinary excretion of folic acid during the period of dosage, give no support to such a conclusion. Conceivably, the active substance in the yeast extract is formed physiologically as a result of *one* of the catalytic functions of folic acid. Thus, a compound could exist which, though able to promote hematopoiesis in a human subject, would not enable the profoundly folic acid-deficient rat to dispense with its multiple requirements for a utilizable form of pteroylglutamic acid. Such an hypothesis would suggest the possibility that the substance might be thymidine or might be involved in the synthesis of thymidine (see sections on folic acid and on mechanism of action).

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Using *L. leichmannii* (ATCC 4797), Collins *et al.* (72) found the average vitamin $B_{12}$ content of milk from cows, sheep, goats and humans to be 6.6, 1.4, 0.12, and 0.41 μg. per liter, respectively. Twelve samples of commercial pasteurized cow's milk averaged 4.0 μg. per liter (range: 1.6–6.5 μg. per liter). In this connection it may be mentioned that the oral administration of cobalt to ewes increased appreciably the vitamin $B_{12}$ content of the milk; the basic mean value of 1.4 μg. per liter was increased from five- to seven-fold by feeding radioactive cobalt (Harper *et al.*, 138). This effect of cobalt may be attributed to the increased microbial synthesis of the vitamin in the rumen (129). On the other hand, it would appear that the requirement of the sheep for cobalt cannot be satisfied by large supplies of vitamin $B_{12}$ (13).

Assays for the vitamin $B_{12}$ content of beef liver and beef skeletal muscle have yielded rather widely varying values. However, the amount of beef muscle often employed daily as a source of extrinsic factor, *i.e.*, about 150 grams, should supply at least 2 μg. and possibly as much as 15 μg. of vitamin $B_{12}$. In this connection, the work of Gardner and associates (116a) may be cited. It was shown that a 70 per cent alcohol extract of beef muscle (10 ml. derived from 400 g.) is essentially inactive hematopoietically, when given *orally* to patients with pernicious anemia in relapse without gastric juice, but definitely effective when given in combination with the gastric factor. Further, it was demonstrated that intravenous injections of the extract (10 ml.) produced the hematologic effects to be expected from the apparent content of vitamin $B_{12}$ (0.37 to 0.9 μg.), as determined microbially. These findings leave no doubt concerning the justification of attributing the extrinsic factor activity of beef muscle at least primarily to vitamin $B_{12}$.

It is of interest to consider the situation with respect to liver, since oral as well as parenteral activity may be extracted from that tissue, and the oral activity is exerted without the aid of gastric juice. Although a half-pound of liver, as used in the original tests of Minot and Murphy (217), should have supplied at least 100 μg. of vitamin $B_{12}$, it is questionable whether it was the vitamin $B_{12}$ contained therein, or derivatives of folic acid in various stages of conjugation, that really were responsible for the hematologic effect of whole liver or orally administered crude liver extracts. Various workers (21, 143, 301) have shown that, in the absence of intrinsic factor, the oral administration of vitamin $B_{12}$ in doses up to 100 μg. or more to patients with pernicious anemia in relapse causes variable and unpredictable hematopoietic effects. With the prolonged administration of such large oral doses of vitamin $B_{12}$ as these, the opportunity for some utilization to occur is very great, even though the supply of intrinsic factor is restricted to a markedly sub-optimal level. However, it is not unlikely that the striking effects originally noted by Minot and Murphy with *orally* administered whole liver or crude liver extracts may be attributed in large part to folic acid, rather than to the anti-pernicious anemia factor (vitamin $B_{12}$) which was isolated subsequently.

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## III. PTEROYLGLUTAMIC ACID (FOLIC ACID; FOLACIN)

In the light of earlier investigations of human blood dyscrasias, the finding in 1945 (82, 221, 305) that synthetic pteroylglutamic acid produces in various megaloblastic anemias of man, in particular, in pernicious anemia, an almost immediate clinical improvement and an increase in the rate of formation of blood cells, for most investigators was quite unexpected. Although previous studies in various avian and mammalian species had indicated that a factor (*e.g.*, vitamin $B_c$ or vitamin M) of then unknown composition was concerned with hematopoiesis, the chemical properties of the substance differed markedly from those of such recognized hematopoietic agents as the extrinsic factor or the anti-pernicious anemia factor. Consequently, the demonstration of the hematologic effectiveness of synthetic folic acid in various types of megaloblastic anemia resulted in a need for definite alteration of previous concepts of hematopoietic mechanisms. Even now, after six years of intensive investigations of the functional interrelationhsip between pteroylglutamic acid and the substance now identified as cyano-cobalamin, a completely satisfactory explanation of the ability of each of these two chemically unrelated compounds to cause a hematologic response in patients with pernicious anemia has not been obtained.

### A. *Synopsis of historical background to the isolation of folic acid*

The story of the many pathways that led to the isolation, identification and eventual synthesis of pteroylglutamic acid has been recounted in many previous reviews (80, 88a, 100, 162, 172, 174, 242, 298, 315, 358, 382). Accordingly, it is considered unnecessary again to present the historical development in great detail. As was the case with many other vitamins, here also a variety of approaches ultimately converged on a single substance or a group of chemically related substances.

1. *Wills factor.* Probably the earliest of these approaches, and one concerning which there is still disagreement, since it has not been incontrovertibly established that folic acid was solely responsible for the effects observed, was that which involved the so-called *Wills factor*. It was reported by Wills in 1931 (372) that a macrocytic anemia observed amongst Hindu women in Bombay, and often but not necessarily associated with pregnancy, responded to an extract of autolyzed yeast, 'Marmite,' to which we have referred previously; this was given in amounts totaling from 8 to 16 grams daily. Later studies (373–379) showed that a similar megaloblastic anemia could be produced in monkeys by feeding a diet patterned after that ingested by the affected human subjects and that the anemia responded either to 'Marmite' or to liver extracts of known activity in the human cases. Since the effect of the liver extracts was convincingly shown not to be due to their content of the classical anti-pernicious anemia factor and because 'Marmite' appears to contain no vitamin $B_{12}$ (*v.s.*), the effect of folic acid and its derivatives on the syndrome observed by Wills assumes great importance. This question perhaps cannot be answered categorically, since no studies with pure pteroylglutamic acid have been reported by Wills; however, other workers in India have found that the syndrome described by Wills responds

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to folic acid. Also, analyses have indicated that the folic acid content of 'Marmite' is in the order of 60 $\mu$g. per gram; this figure was obtained both by assay in rats (187) and by microbial assays after suitable enzymatic treatment to release the free folic acid from the conjugated form characteristic of yeast (383). It would appear, therefore, that 'Marmite,' as used by Wills and her associates, may have supplied from 0.5 to 1.0 mg. of folic acid daily. Since this amount of folic acid is equal to or greater than the estimated daily requirement (82) and is no larger than that frequently found to be markedly effective in initiating a hematologic remission in pernicious anemia (80), the folic acid content of 'Marmite' is compatible with the concept of the identity of the 'Wills factor' and this vitamin. We believe, therefore, that the evidence favors greatly the conclusion that folic acid was responsible for the hematologic response observed by Wills and her coworkers.

In fairness it should be pointed out again, however, that several investigators have accepted the hypothesis that the hematologic effect of extracts of autolyzed yeast is to be attributed to the presence of an unrecognized hematopoietic agent. In support of such a possibility may be cited the observation that, despite the apparent absence of vitamin $B_{12}$, the response to oral administration of extracts of autolyzed yeast is augmented by the simultaneous administration of normal human gastric juice (see also Section II. G. 8).

2. *Vitamin M*. The investigations in monkeys, carried out by Wills and her associates (373–379), in which a megaloblastic anemia developed on diets nearly identical with those consumed by the human subjects, almost certainly represented a study of folic acid deficiency in this species. Thus, extensive studies by Day and coworkers in Arkansas and by others (see review by Day, 88a) have shown that a deficiency syndrome in monkeys, characterized by progressive anemia and leucopenia, lesions of the oral cavity, and diarrhea, and apparently identical with that described by workers in India, can be relieved completely by sources of vitamin M or by synthetic pteroylglutamic acid. Although earlier conclusions did not agree with this statement, it is now evident that microbial assays for folic acid were not adequate at first to measure the total folic acid content of natural materials shown to have vitamin M activity (since a large proportion of the vitamin exists in bound forms which, as such, cannot be utilized by the majority of microorganisms).

3. *Growth and hematopoiesis in the chick*. Other prominent historical features of the development of folic acid that should be mentioned include the studies of a component of yeast (factor U) which promoted the growth of chicks (319). Nutritional anemia in chicks was employed by others to guide the isolation in 1942 of vitamin $B_c$ in the free and later in the conjugated form as obtained from yeast (242). As with the identification of vitamin M, complications were encountered concerning the interpretation of the activity in chicks of concentrates containing conjugated forms as well as free folic acid. These difficulties led to confusion and to the introduction of the terms factor R, vitamin $B_{10}$ and vitamin $B_{11}$; it is now agreed, however, that the effects of these materials can be duplicated in chicks by either free or conjugated pteroylglutamic acid.

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4. *Microbial assays.* Most rapid progress in the isolation of pteroylglutamic acid came with the use of assays in which microorganisms unable to satisfy their need for folic acid by synthesis were employed. The earliest work in this direction, and that which proved to be of basic importance in many subsequent studies, was that of Snell and Peterson (297); this showed that *Lactobacillus casei* (ATCC 7469) requires for growth a substance found in liver and other natural materials which could be adsorbed on and eluted from charcoal. For this reason it was referred to as the *norite eluate factor*, and also as the *L. casei factor;* this substance was isolated from both liver and yeast in 1943 (174). Evidence that this factor, required by *L. casei*, also was needed by the chick, was presented (161), and it soon became evident that vitamin $B_c$ and the *L. casei* factor were identical. Additional comments on the problems encountered in microbial assays are made in a later section.

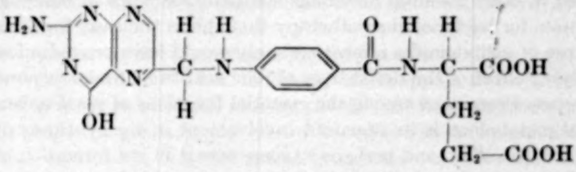
### *B. Nomenclature of pteroylglutamic acid*

The term, "folic acid," was introduced by Mitchell, Snell and Williams (220) as a designation for material in green plants (*e.g.*, spinach) that is utilized for growth by the microorganism, *Streptococcus lactis* R (now referred to as *S. fecalis* R, ATCC 8043). The substance responsible for this microbial activity apparently was not obtained in pure crystalline form and it is now known that this organism also can utilize pteroic acid and $N^{10}$-formylpteroic acid (the *SLR factor*) (184), as well as pteroylglutamic acid and its derivatives, *e.g.*, the citrovorum factors (to be discussed later). *S. fecalis* R is one of relatively few organisms (311) which, although unable to synthesize folic acid, has the capacity of converting pteroic (or $N^{10}$-formyl-pteroic) acid into compounds utilizable for growth, presumably folic acid and the citrovorum factor. The fact that the term folic acid is now applied generally to pteroylglutamic acid, both in this country and abroad, is an excellent example of the result likely to follow when a euphonious name is prematurely applied to a biological effect as if it were known to be due to a single compound. Acceptance of such a name often results, regardless of considerations of priority, or of the structures ultimately shown to be responsible.

In an attempt to introduce a term less susceptible to criticism a joint committee on nomenclature of the American Institute of Nutrition and the American Society of Biological Chemists recommended, and the respective societies approved, the introduction of the new term *folacin*, as a synonym for pteroylglutamic acid. However, although used by some authors, the new name has not found wide-spread acceptance.

### *C. Structure and synthesis of pteroylglutamic acid*

Although the new factor (vitamin $B_c$; *L. casei factor*) had been isolated from two sources, liver and yeast, only very scanty supplies of the pure compound could be made available in this manner for the degradation work that is indispensable for the elucidation of molecular structure. Rapid advance occurred, however, when it was found that, during the growth of a species of *Corynebacterium*, a derivative of folic acid is produced (162). Relatively large amounts of so-called *fermentation folic acid* were provided in this manner by the large scale aerobic growth of this diphtheroid organism. Degradation studies led to the formulation of the structure of the derivative as a tetrapeptide composed of 3 moles of (γ-linked) glutamic acid and 1 mole of *p*-aminobenzoic acid, while the liver *L. casei* factor, which, in the racemic form, could be obtained from the tetrapeptide, was shown (174) to be:



*Pteroylglutamic acid*

For additional information concerning the studies of the degradation of vitamin $B_c$ and the conjugated form of yeast, pteroyl-hexa-γ-glutamylglutamic acid, often termed the "heptaglutamate" (Parke, Davis group); liver *L. casei* factor, pteroylglutamic acid, and "fermentation folic acid," pteroly-γ-glutamyl-γ-glutamyl-glutamic acid, often termed the "triglutamate" (American Cyanamid group); and the SLR factor, $N^{10}$-formylpteroic acid (Merck group); the reader is referred particularly to the reviews of Hutchings and Mowat (162) and Jukes and Stokstad (174).

Synthesis of pteroylglutamic acid, N-[4-{[(2-amino-4-hydroxy-6-pteridyl)-methyl]amino}benzoyl]-L(+)glutamic acid, was first accomplished in cooperating divisions of the American Cyanamid Co. (3) by appropriate reaction together of 2,5,6-triamino-4-hydroxy-pyrimidine, 2,3-dibromopropionaldehyde and p-aminobenzoyl-L(+) glutamic acid. A number of alternative methods of preparation also have been reported.

The demonstration that the pteroic acid moiety of folic acid is a derivative of *p*-aminobenzoic acid offered a logical basis for the observed action of sulfonamides in inhibiting the synthesis of folic acid by bacteria both *in vitro* and in the intestinal tract of animals. It is generally accepted that this is the explanation of the effectiveness of the oral administration of such compounds as sulfanilylguanidine (29), succinylsulfathiazole (356, 368), and other antagonists of *p*-aminobenzoic acid in favoring the development of a deficiency of folic acid in certain species (*e.g.*, the rat) fed *purified* diets containing no significant amount of folic acid or its utilizable derivatives. The fact that rats fed such purified diets (without sulfonamides) are able to survive and to grow, while the addition of sulfonamides, particularly to the diet of weanling animals, leads to the development of manifestations of a severe deficiency of folic acid, is reasonably interpreted as signifying that the requirement of the rat for folic acid under ordinary circumstances can be satisfied by the synthetic activity of certain sulfonamide-sensitive symbiotic intestinal microörganisms. That man also can obtain, in a similar manner, sufficient amounts of folic acid or its derivatives to survive under ordinary conditions, is thought to be likely, but this apparently has not been demonstrated experimentally.

Since the inhibitory effect of sulfonamides on various susceptible microorganisms, which is competitively antagonized by *p*-aminobenzoic acid, is but rarely (and non-competitively) prevented by *highly purified* folic acid, it is evident that, in addition to the synthesis of folic acid, *p*-aminobenzoic acid must be involved in other essential metabolic synthetic reactions of bacteria. Indeed, it is fortunate for bacterial chemotherapy that this is the case, for otherwise the practical use of sulfonamides almost certainly would have proved infeasible due to a nullifying effect of the derivatives of folic acid to be found in normal mammalian tissues. Prominent among the essential functions of *p*-aminobenzoic acid in *bacterial* metabolism is its apparent involvement in the synthesis of vitamin $B_{12}$ by *E. coli* (87, 279), and perhaps to some extent in the formation of a newly recognized essential metabolite, *p*-hydroxybenzoic acid (86); it is not unlikely that it is also involved in other essential reactions.

### *D. Chemical determination of folic acid derivatives*

The presence of a *p*-aminobenzoic acid moiety in pteroylglutamic acid affords a means of determining it chemically (163); the method is described by the U.S.P. XIV. In essence, it consists in the hydrolytic and reductive (zinc) cleavage of the molecule to yield a pteridine and *p*-aminobenzoylglutamic acid. The latter is determined colorimetrically or spectrophotometrically, after diazotization and coupling with N-(1-naphthyl)ethylenediamine, by the method developed for the determination of sulfonamides (34). A modification of the procedure has been described (122) in which reduction is accomplished through the use of titanous chloride instead of zinc; this has the advantage of avoiding interference from adenine, and of yielding much lower blanks with tissues.

### *E. Requirements for folic acid by various species*

1. *Rats and other mammals.* In the *young rat* fed a purified diet containing a sulfonamide, a deficiency of pteroylglutamic acid usually is manifested first by a diminution in the rate of growth, and subsequently by a loss of weight which soon is accompanied by marked diarrhea and a leucopenia that involves the granulocytic series. Anemia is less commonly seen since death is likely to occur for other reasons before the number of circulating red blood cells can be reduced markedly due to the deficiency of folic acid. In addition to the manifestations noted, Asenjo (8) has commented on the frequent occurrence of splenic infarcts. Also a possible role of folic acid in pigment formation is suggested by the development of gray hair in black rats fed a sulfonamide-containing purified diet. However, this manifestation may be secondary to a deficiency of pantothenic acid, since studies by Wright and Welch (397) indicated that pantothenic acid is not utilized properly by folic acid-deficient rats.

Extensive studies of folic acid deficiency in *swine* have been made by several groups, particularly that of Wintrobe, Cartwright and co-workers (54, 55, 56, 386) and that of the reviewers and their associates (146, 147, 148, 363, 364).

Since these studies will be discussed more fully in a later section, it will suffice for the present to state that a deficiency of folic acid in the pig is characterized particularly by an interference with growth, and by the development of diarrhea and macrocytic anemia with megaloblastic hyperplasia of the bone marrow.

In an earlier section the need for folic acid which has been demonstrated in the monkey was discussed. Other mammals whose requirement for folic acid has been investigated include mice, dogs, guinea pigs, foxes and mink (see review by Jukes and Stokstad, 174).

2. *Avian species.* In avian nutrition pteroylglutamic acid has been shown to play a very important role. Thus, in chicks, which appear to be far less capable than rats of deriving an adequate amount of folic acid from materials synthesized by intestinal microorganisms, the use of an otherwise adequate purified diet leads to the suppression of growth, feathering, and, as previously mentioned, disturbances in hematopoiesis (242). The addition of succinylsulfathiazole to such diets has little or no tendency to increase the requirement for folic acid (which approximates 0.5 to 1.0 mg. per kg. of diet). The occurrence of perosis, frequently seen in chicks inadequately supplied with any one of a variety of factors required for growth, has been observed also in folic acid deficiency (79).

Of great general significance was the discovery by Hertz and Sebrell (153) that folic acid is essential for the response of the chick oviduct to estrogenic stimulation. In subsequent studies by Hertz (150, 151, 152, 154) it was shown that folic acid also is essential for tissue growth in response to estrogen in the genital tract of the rat and the monkey. Presumably, a number of the cellular components of tissues which are susceptible to estrogenic stimulation cannot be synthesized in the absence of an essential catalyst derived from folic acid. On the other hand, although they confirmed the results with estrogen, Dorfman and associates (89) found that the response of susceptible tissues to androgens was not interfered with by folic acid deficiency.

Other avian species whose requirement for folic acid has been investigated include turkeys (254) and ducks (142). The larvae of various insects also have been shown to require folic acid (107) and a protozoan species, *Tetrahymena geleii*, with a similar requirement, has been studied extensively (185).

### *F. Enzymic release of folic acid from polyglutamic acid derivatives ("conjugates")*

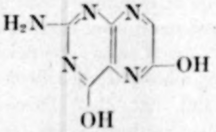
Since the bacteria used in assaying for folic acid cannot respond to the "heptaglutamate," pteroyl-hexa-$\gamma$-glutamylglutamic acid, found in yeast, liver and other tissues, it has been necessary to devise methods for obtaining maximal liberation of "free" folic acid. For this and other reasons various studies of the properties and the purification of enzymes ("conjugases") that hydrolyze these conjugated forms of folic acid have been carried out. There appear to be two main types of such enzymes; one of these, the pH optimum of which, under the conditions studied, is approximately 7, has been studied extensively by Laskowski and associates (77, 182, 192, 214). This type of enzyme is widely distributed, and accounts for the "conjugase activity" of liver at pH 7 (216, 365), but is often referred to as "chicken pancreas conjugase," from which tissue it usually has been prepared. The highly purified enzyme, which has been shown to require calcium as a coenzyme, yields not pteroylglutamic acid but pteroyl-$\gamma$-glutamylglutamic acid as an end-product of the cleavage of either the "triglutamate" or the "heptaglutamate". The enzyme will attack, but less rapidly, the alpha-linkage of pteroyl-$\alpha$-glutamyl-$\alpha$-glutamylglutamic acid, which so far as is known does not occur naturally (77).

The second class of enzyme (216) is composed, according to Mims and Bird (213), of two separate enzymes, neither of which, by itself, is able to release "free" folic acid; however, no other studies in support of this claim have appeared. This enzyme system occurs in many tissues, particularly liver, kidney and intestine, but hog kidney is commonly used for the preparation of active extracts (26, 27). As ordinarily employed, the optimal pH for the cleavage of conjugated folic acid is approximately 4.5. It was reported (215) that nucleic acids are highly inhibitory to this type of conjugase; the inhibition was attributed to combination with essential sulfhydryl groups, since this type of inhibition, and that of simple compounds known to react with SH-groups, could be removed by the addition of either cysteine or hydrogen sulfide. Hill and Scott (158) have shown that relatively high levels of cysteine are required to produce maximal activation of the enzyme and have demonstrated the practical value of the use of cysteine, in connection with the enzyme, in the preparation of samples for the microbial determination of "total" folic acid. This type of enzyme releases free pteroylglutamic acid from the "heptaglutamate," while the other product of the enzymic cleavage is considered to be a glutamic acid polypeptide composed of six-$\gamma$-linked units (241).

In view of the findings (22, 144, 329, 361, 362) which indicate that utilization of the "heptaglutamate" may be seriously disturbed in pernicious anemia, and that orally ingested inhibitors of conjugase activity may contribute to the inefficient utilization of the conjugated form of folic acid (22, 329, 330), further study of conjugase activity in normal and pernicious anemic individuals is indicated. This subject receives additional consideration in a later section.

### *G. Relation of xanthopterin to the metabolism of folic acid*

Xanthopterin, a substance which occurs naturally in the wings of certain yellow butterflies (117) and, in a combined form, in human urine (188), has a structure very closely related to that of the pteridine portion of pteroylglutamic acid. The urinary elimination of a "pro-xanthopterin" is increased following the administration of folic acid (102).



*Xanthopterin*

It has been reported that xanthopterin will relieve the anemia induced in rats by feeding a diet of goat's milk (339) and will induce leucocytosis and stimulate growth in rats fed a purified diet containing succinylsulfathiazole (335). Various reports from Norris and Majnarich (231, 232) claiming an hematopoietic effect of the pterin in rats appear not to have been soundly based (248). In fact, there has been rather consistent failure to confirm the various suggestions of a folic acid-like action of xanthopterin in this species (9, 78, 396) and further interest in the compound might not be justified were it not for other evidence of its biological activity. Cerecedo and his associates (219, 285) reported that xanthopterin, like folic acid, causes a significant improvement in the lactation performance of mice (and rats) fed purified diets, while Totter and co-workers (338) observed hematopoietic responses to synthetic xanthopterin in folic acid-deficient monkeys. Also, in the laboratories of the writers, each of two folic acid-deficient, macrocytically anemic swine showed a small but definite hematologic response to parenterally administered xanthopterin (10 mg. daily for 10 days) (148), and, subsequently, a third animal responded to a similar dose of xanthopterin-6,7-$C^{14}$. Cartwright and his associates (55) noted a slight but definite reticulocytosis (9 per cent) in a similarly deficient, anemic pig given a single injection of 20 mg. of xanthopterin; however, other of their animals failed to respond. Even in one patient with pernicious anemia in relapse a small reticulocyte response appeared to follow the administration of xanthopterin (144). It has been reported that anemia in Chinook salmon responds to injections of xanthopterin (233, 287), while the larvae of the insect *Ephestia kuehniella* utilize xanthopterin as a substitute for folic acid, although the pterin is required in a concentration of about 1000 times that of the vitamin (107).

A possible clue to some of these actions of xanthopterin may be found in studies (365, 395, 396) of a curious effect of xanthopterin on the release of folic acid (or its microbially active derivatives) by hepatic tissue incubated *in vitro*. Thus, it was shown (365) that addition of the pterin to homogenates of normal swine liver can increase by as much as ten-fold the amount of *L. casei* factor activity formed during incubation. It was suggested that the liver contains a precursor of pteroylhexaglutamylglutamic acid which is susceptible to attack competitively by two types of enzyme released by homogenizing the tissue; one reaction leads to the formation of *L. casei* factor and the other to a compound having no demonstrable biological activity. Xanthopterin may function *in vitro* by blocking the reaction leading to inactivation, with a resultant marked increase in the biologically active material found at the end of the period of incubation. If this explanation of the effect of xanthopterin proves to be correct, the true amount of pteroylglutamic acid derivatives in liver is much higher than that observed following ordinary methods of release and determination.

The possibility that xanthopterin is converted to folic acid by liver enzymes was entertained (396), but recent work in these laboratories, using xanthopterin labeled with radioactive carbon in positions 6 and 7 (4), appears to have eliminated this possibility (6). Administration of the labeled pterin to a folic acid-deficient pig had a small hematopoietic effect, but radioactivity could not be demonstrated in folic acid isolated from the liver (with the aid of carrier vitamin.) It should be mentioned that only about 5 per cent of the hepatic material with activity for *L. casei* behaved like citrovorum factor, a finding which justified the use of folic acid as a carrier. Further, the incubation of an homogenate of normal swine liver *in vitro* with xanthopterin-6,7-$C^{14}$ did not lead to the appearance of the labeled carbon in folic acid isolated from the homogenate. It may be that even in folic acid-deficient animals there is, in some cases at least, a sufficient amount of stored conjugated forms of the vitamin, the major pathway of the metabolic handling of which leads to inactivation, that xanthopterin, by interfering with this inactivation, can favor the formation of utilizable derivatives of the vitamin. However, additional work is needed to explain the intriguing effects of xanthopterin on folic acid metabolism, hematopoiesis, lactation and growth.

### H. *Metabolic alteration of folic acid to form the citrovorum factor (folinic acid; leucovorin)*

In earlier sections of this review we have referred to the *citrovorum factor*, which also has been termed *folinic acid* (10, 30). In its synthetic form the compound has been called *leucovorin* (42) and *folinic acid*-SF (206, 247) (the letters SF presumably referring to "synthetic form").

It was postulated by Sauberlich and Baumann (269, 270) that a factor required for the growth of the microorganism, *Leuconostoc citrovorum* (ATCC 8081), was related in structure to folic acid. Additional evidence in favor of this relationship was offered by Sauberlich (268), who showed that the urinary elimination of the factor by rats and man was increased following the administration of pteroylglutamic acid. That the citrovorum factor can be derived enzymatically from folic acid was established by Nichol and Welch (229) who showed that liver slices, *in vitro*, will convert synthetic pteroylglutamic acid to the new factor. Synthesis, together with various chemical studies (37, 206, 247, 281), finally established the constitution of the compound and indicated its close similarity to folic acid.

Guided by microbial assays, the factor was concentrated by several groups (5, 39, 269, 270), liver being employed in the majority of instances, although the Cleveland group (5, 366) used the urine of human subjects ingesting large doses of folic acid.

1. *Role of ascorbic acid.* In connection with the program of concentration of the factor from urine it was noted that considerable variation occurred in the amount of citrovorum factor excreted by individuals ingesting constant daily doses of synthetic folic acid. One important cause of these variations appeared to be the level of the dietary intake of ascorbic acid. Although vitamin C by itself had no effect on the urinary excretion of citrovorum factor (268), in conjunction with folic acid it caused an increase of from two- to four-fold in the excretion of the metabolite of pteroylglutamic acid (5, 359, 366). Studies *in vitro*, using the liver tissue of rats, pigs or chicks, showed (229) that ascorbic acid similarly augmented the enzymatic conversion of folic acid to citrovorum factor, although such

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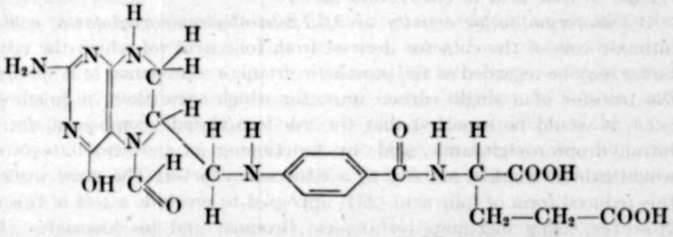
powerful reducing agents as glutathione and cysteine exerted little or no effect. On the other hand, the ascorbic acid analogue, glucoascorbic acid, was able to facilitate the conversion both *in vitro* (229) and *in vivo* in rats (359, 366). These findings led to the suggestion that ascorbic acid may function as a hydrogen donor in the conversion of folic acid to the citrovorum factor, which was regarded as a reduced form of the parent compound (229). This view is in agreement with present concepts of the structure of the synthetic material.

2. *Structure of the citrovorum factor.* Studies recently reported by Shive and his associates (206, 247), indicate that the citrovorum factor (folinic acid) is a formylated tetrahydrofolic acid. Although earlier descriptions of the methods of synthesis used by the Texas group (281) were not entirely clear, the definitive techniques used by Brockman *et al.* (37), May, *et al.* (206), and Pohland, *et al.* (247) give a clear picture of the formation of the new factor from folic acid. Also, these papers emphasize the importance of reductive steps in the conversion of pteroylglutamic acid to the citrovorum factor.



*Synthetic citrovorum factor (folinic acid-SF; leucovorin)*

Both groups have used N$^{10}$-formyl-pteroylglutamic acid as a precursor; the transfer of the formyl group from position-10 to position-5, through the intermediary formation of an imidazoline derivative, and the loss of the formyl group from position-5 (with inactivation) under the influence of acid, are postulated (206, 247). Such "wandering" of the formyl group may account for the occurrence in nature of the SLR factor, demonstrated to be N$^{10}$-formyl-pteroic acid (387). It should be appreciated that the naturally occurring citrovorum factor or factors may not be identical in structure with the synthetic material (286a).

3. *Enzymatic alteration of folic acid.* The enzymatic conversion of folic acid to the citrovorum factor *in vitro* originally was accomplished with liver slices incubated aerobically (229); subsequently, a cell-free system which contains all the essential components of the conversion system and which functions efficiently under anaerobic conditions has been obtained from liver (226). Studies directed toward the purification of the components of the system are in progress.

Although extensive studies of the distribution of the enzyme system that converts folic acid to the citrovorum factor have not been made, it appears that liver and kidney are particularly active. In view of the findings of Lajtha (189, 190) in which folic acid, as well as citrovorum factor, was shown to cause disappowerful reducing agents as glutathione and cysteine exerted little or no effect. On the other hand, the ascorbic acid analogue, glucoascorbic acid, was able to facilitate the conversion both *in vitro* (229) and *in vivo* in rats (359, 366). These findings led to the suggestion that ascorbic acid may function as a hydrogen donor in the conversion of folic acid to the citrovorum factor, which was regarded as a reduced form of the parent compound (229). This view is in agreement with present concepts of the structure of the synthetic material.

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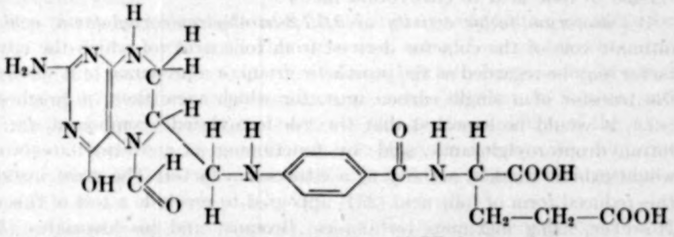

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pearance of megaloblasts from pernicious anemic bone marrow *in vitro*, it is evident that the enzyme system should be found in that tissue, if the citrovorum factor is the active form of folic acid. Although human tissues have not yet been studied, suspensions of normal rabbit bone marrow exhibited a small but definite increase in citrovorum factor activity when incubated anaerobically with synthetic folic acid. Thus, it is evident that the "activation" of folic acid, although probably occurring primarily in liver and kidney tissue, can take place in at least some of the sites of utilization of the active form of the vitamin.

It may be assumed that the conversion to the structure assigned to folinic acid-SF involves at least two and possibly more reactions catalyzed by enzymes; these reactions involve the reduction of the double bonds in positions 5-6 and 7-8, and the introduction of the formyl group into position-5. Since $N^{10}$-formyl folic acid exhibited no more, and possibly less, activity than folic acid in the conversion system (229), it appears likely that this compound, which serves as a precursor in the chemical synthesis, is not a biological intermediate in the conversion of folic acid to citrovorum factor.

4. *Citrovorum factor activity of 5,6,7,8-tetrahydropteroylglutamic acid.* If the ultimate role of the cofactor derived from folic acid (of which the citrovorum factor may be regarded as the prosthetic group or a precursor of it) is to catalyze the transfer of a single carbon unit, for which view there is much evidence (*v.i.*), it would be expected that the "de-formylated" compound, *i.e.*, 5,6,7,8-tetrahydropteroylglutamic acid, by functioning as a "formyl-acceptor," also would exhibit marked activity as a citrovorum factor. The great instability of this reduced form of folic acid (234) appeared to preclude a test of this concept. However, using ingenious techniques, Broquist and his associates (38) very recently have proved that the tetrahydro-compound indeed is biologically active, not only as a growth factor for *Leuconostoc citrovorum*, but in other respects. This demonstration adds a further link to the chain of evidence indicating that folic acid is the precursor of a factor the function of which is to transfer the −CHO group in various metabolic processes, *e.g.*, in the synthesis of thymine, purines, serine, and of the labile methyl group.

### *I. Significance of citrovorum factor to the microbial determination of "folic acid"*

The organisms most commonly used for the determination of folic acid, namely, *Lactobacillus casei* (ATCC 7469) and *Streptococcus fecalis* (ATCC 8043) respond also to the citrovorum factor (37). This is not surprising since it is now reasonable to assume that any form of life able to utilize pteroylglutamic acid must be able also to convert it to its functional derivatives. From the practical standpoint, however, it signifies that previously published data concerning the pteroylglutamic acid content of natural materials must be regarded as including also the "activated" form. Actually, it has been known for a long time that values for "folic acid" obtained with these two organisms could include more than pteroylglutamic acid. Thus, *S. fecalis* responds to pteroic acid and the SLR factor, while *L. casei* effectively utilizes the "triglutamate" (pteroyl-γ-glutamyl-γ-glutamylglutamic acid). Further to complicate the picture, it may be mentioned that the "diglutamate," pteroyl-$\gamma$-glutamylglutamic acid, is utilized in a manner indistinguishable from that of pteroylglutamic acid. That this compound also occurs naturally is indicated by the work of Laskowski and associates (77, 182), who noted that it is the end-product of the hydrolysis of the "triglutamate" at neutral pH by the conjugase which is found in chicken pancreas (and other tissues), and a similar observation was made by Pfiffner (240) using the "heptaglutamate" and the same enzyme.

Although none of these microorganisms can utilize the "heptaglutamate" for growth, its presence in materials to be assayed may offer a serious complication. This is because the response of microorganisms, particularly *L. casei*, to pteroylglutamic acid is depressed rather erratically by the polyglutamate, and this fact should be taken into account when assays for "free" folic acid must be carried out in the presence of "conjugate." Among the microorganisms so far studied only *Tetrahymena geleii* is able to utilize the heptaglutamate, but this organism has been little used for assay because of the difficulties encountered in supplying its other requirements.

According to the best information available at the present time, it should be possible to determine with reasonable accuracy, using *Leuconostoc citrovorum* (ATCC 8081), that proportion of the "free folic acid" which exists as the 5-formyl derivative of 5,6,7,8-tetrahydropteroylglutamic acid (citrovorum factor), and certain functional derivatives of it. In view of the great instability of the compound in the "deformylated" state (38), it is unlikely that assays, as ordinarily carried out, would measure any appreciable proportion of material originally present in this form.

In conducting differential determinations of the relative proportions of the "folic acid" present as citrovorum factor, it is imperative that most careful measurements of the "standard response" of each organism to each pure compound be made under rigidly controlled conditions. Recently, Swenseid and associates (328) concluded that ". . . . the folic acid activity of mouse liver is due to its folinic acid content. Under standard dietary conditions, folic acid is absent from the liver or present in very small amounts." Since the reference standard used in the measurements of folinic acid activity in this investigation consisted of a synthetic material "of unknown purity," this conclusion will require confirmation. However, from paper chromatographic and bioautographic studies of "liver digest," May and associates (206) have concluded that "folinic acid, and a slower moving factor not identical with folic acid constitute the major part of the principles of liver which promote the growth of *Lactobacillus casei* in place of folic acid." These workers suggest that folic acid which has been isolated from liver actually may represent material derived during the isolation process from folinic acid and related factors.

It should be pointed out also that the citrovorum factor in fresh liver, and presumably in most other tissues, exists as a conjugate which behaves in all respects as a polyglutamate analogous to that of pteroylglutamic acid (157). Since *Leuconostoc citrovorum* cannot utilize conjugated forms of the citrovorum factor known to occur in natural materials, it is essential in conducting microbial assays for this material, as it is in determining folic acid, that hydrolytic cleavage of the conjugated form be as nearly complete as possible.

Care must be taken, as with the determination of vitamin $B_{12}$, to avoid erroneous results due to the presence of the desoxyriboside of thymine (thymidine), a compound which will promote the maximal growth of *Leuconostoc citrovorum* in the absence of the citrovorum factor (268). This complication is not a serious one, since the citrovorum factor causes rapid growth, permitting assays to be made in about 20 hours, while the growth induced by thymidine is not apparent until after a lag period which is greater than 20 hours in length. With *S. fecalis*, in media containing purines, but not folic acid, maximal growth can be obtained with either thymine or its desoxyriboside, while with *L. casei*, under these conditions, the growth which can be obtained with these substances is equivalent to about half that obtainable with folic acid or citrovorum factor.

### J. *Ability of certain steroids to cause microbial growth in the absence of folic acid and its derivatives*

Dehydroisoandrosterone acetate in relatively large amounts is able to promote the growth of *S. fecalis* R and *L. casei* in the absence of folic acid (115), while cortisone acetate, in addition to having these effects, has been shown to support the growth of *Leuconostoc citrovorum* in the absence of citrovorum factor (114, 114a). An explanation of these very intriguing findings is greatly needed.

### K. *Mechanism of action of analogues of folic acid (in particular, 'Aminopterin' and its derivatives)*

Structural antagonists of biologically active compounds have served as exceedingly useful tools in exploring biological mechanisms (258, 357, 393). Occasionally, they have proved to have important therapeutic applications; the outstanding example of such utility, of course, is to be found in the sulfonamides. Another class of metabolite antagonists, which has served therapeutically to alter significantly the course of certain leukemias, particularly in children, includes various analogues of folic acid, in particular, 'Aminopterin,' in whch the hydroxyl group in position-4 of pteroylglutamic acid is replaced by an amino group. The $N^{10}$-methyl derivative of 'Aminopterin,' often termed 'A-methopterin,' and several other analogues also have proved useful clinically.

These compounds have appeared to differ in their action from other examples of metabolite antagonists in that, in higher animals, at least, they have been reversed in their effects to only an exceedingly limited degree by the compound the structure of which they so closely resemble and the function of which they unquestionably block, *i.e.*, folic acid (109, 112, 156, 236, 237, 243, 256, 334). Indeed, so restricted is folic acid in its ability to prevent or reverse the action of 'Aminopterin' that consideration was given to the possibility that the analogue fortuitously interfered also with the function of other essential metabolites. For example, a normal 150-gram rat given injections of only 25 μg. of 'Aminopterin' daily will die within about seven days despite the concurrent daily injection of 5 mg. of folic acid (230). Ordinarily, it is possible to demonstrate a protective action of folic acid only by previous saturation of animals with folic acid, and by using otherwise barely toxic doses of 'Aminopterin ' This essential failure of folic acid as a reversing agent is of considerable practical significance in that leukemic patients treated with the analogue can become exceedingly ill as a result of the profound need for functional derivatives of folic acid, and until recently there has been no means of ameliorating these manifestations of toxicity (354).

In view of the demonstrated enzymatic conversion of folic acid to a metabolically active derivative, it is logical to expect that 'Aminopterin' might block one or more of the enzyme-catalyzed reactions required for the "activation" of the vitamin. Accordingly, studies of this possibility were made by Nichol and Welch (230). It was found (a) that 'Aminopterin' added to liver slices effectively blocks the conversion of folic acid to citrovorum factor; (b) that the liver of 'Aminopterin'-treated rats cannot effect the conversion; (c) that the urinary excretion of citrovorum factor by rats, following the administration of folic acid, is almost completely blocked by giving minute doses of 'Aminopterin'; and (d) that the product of the conversion, namely, the citrovorum factor, effectively reverses the toxic action of the analogue *in vivo*. The finding that the effects of 'Aminopterin' or its $N^{10}$-methyl-derivative can be prevented or reversed by citrovorum factor has been made independently by Brockman *et al.* (37) and by others (40, 41, 42, 46).

Some workers (45) have interpreted the reversal of the toxic effects of 'A-methopterin' by citrovorum factor as signifying that the 4-amino derivatives of folic acid act primarily as antagonists of citrovorum factor; the correctness of this interpretation perhaps depends on the point of view (366). Certainly the lethal effects of the 4-amino analogues of folic acid ultimately must be attributed to their competitive inhibition of the utilization of citrovorum factor, while protection against 'Aminopterin' can be achieved with ratios of leucovorin to antagonist of 1:1 to 10:1 (230). However, these antagonists interfere so profoundly, and essentially *non-competitively*, with the utilization of folic acid that it is tempting to regard this site of action as the predominant one, even though this inhibition is not a lethal one *if the organism can obtain citrovorum factor from its environment.*

Presumably the basis for the remarkable potency of this class of analogues as antagonists of folic acid is to be found in an extremely high affinity which the analogues possess, due to a change in properties resulting from the replacement of the 4-hydroxyl by the 4-amino group, for an enzyme the normal substrate of which is folic acid or a compound lying intermediate between folic acid and the citrovorum factor (possibly a dihydrofolic acid).

Due to the postulated high affinity of the analogue for susceptible enzymes, as compared with the normal substrate, it is suggested that 'Aminopterin' diminishes the metabolic alteration of folic acid to a biologically negligible level and, within the range of concentrations of folic acid attainable under physiological conditions, little displacement of the analogue from the enzyme can be obtained by the use of pteroylglutamic acid. Even with bacteriologic media in which the relative concentrations of analogue and metabolite are much more subject to control, the effectiveness of folic acid as a reversing agent diminishes rapidly as the concentration of 'Aminopterin' is increased. Thus, using *S. fecalis* R (237), half-maximal growth was obtained with a concentration of 0.003 μg. of folic acid per ml. when the concentration of 'Aminopterin' was 0.008 μg. per ml.; however, with only a 2½-fold increase in the concentration of the antagonist (0.02 μg. per ml.), the concentration of folic acid necessary to obtain half-maximal growth was increased 100-fold (0.3 μg. per ml.). On the other hand, as might be expected, the toxic effects of 'Aminopterin' on microorganisms can be antagonized readily by citrovorum factor. Using *Leuconostoc citrovorum*, Sauberlich (268) observed such an interference, and Broquist and associates (41) have presented data which show that the antagonism is strictly competitive. Also with *S. fecalis* the toxic effects of 'Aminopterin' were much more effectively blocked by citrovorum factor than by folic acid (41).

It is evident, therefore, that 'Aminopterin' exerts two quite different types of antagonism. That which is related to the citrovorum factor is competitive and readily reversible, while that concerned with folic acid is essentially non-competitive and only reversible under conditions where very high concentrations of folic acid are attainable. Obviously, therefore, the practical means of attempting to reverse the toxic actions of the 4-amino analogues of folic acid involve the use of citrovorum factor, but the *principal* reason for the development of the toxic effects of the analogues when folic acid is administered is the blockade of the biological utilization of the vitamin.

In discussing the mechanism of action of the 4-amino-analogues of folic acid, Broquist and associates (38) have suggested that the antagonists may undergo preferential enzymatic alteration in a manner comparable to the conversion of folic acid to the citrovorum factor. It was proposed that the 5—CHO group acquired in this way may then react with the 4-amino group to form a stable ring, thus preventing the transfer of the formyl group. However, such metabolic alteration of the analogue, although it might effectively "trap" one-carbon units, eventually should lead to complete inactivation of the antagonist and, thus, to "escape" from its toxic action, unless the postulated new derivative of the 4-amino analogue were to behave as a more potent antagonist of the enzymatic alteration of folic acid than the parent compound. Evidence for a metabolic alteration of 'Aminopterin' could not be obtained by Nichol and Welch, using rat liver slices (230). Although the reviewers (360) have noted apparently *bona fide* reticulocyte responses to 'Aminopterin' in folic acid-deficient, anemic swine, this observation would suggest that this species can replace the 4-amino group with an hydroxyl group to form folic acid. A similar observation was made by Thiersch (333) who found that swine can tolerate relatively enormous doses of 'Aminopterin' without developing the toxic manifestations characteristically seen in most species.

Studies comparable to those carried out with the 4-amino analogues have not been made with other compounds shown to have activity as biological antagonists of folic acid. It may be assumed, however, that the less markedly active analogues, relative concentrations of analogue and metabolite are much more subject to control, the effectiveness of folic acid as a reversing agent diminishes rapidly as the concentration of 'Aminopterin' is increased. Thus, using *S. fecalis* R (237), half-maximal growth was obtained with a concentration of 0.003 μg. of folic acid per ml. when the concentration of 'Aminopterin' was 0.008 μg. per ml.; however, with only a 2½-fold increase in the concentration of the antagonist (0.02 μg. per ml.), the concentration of folic acid necessary to obtain half-maximal growth was increased 100-fold (0.3 μg. per ml.). On the other hand, as might be expected, the toxic effects of 'Aminopterin' on microorganisms can be antagonized readily by citrovorum factor. Using *Leuconostoc citrovorum*, Sauberlich (268) observed such an interference, and Broquist and associates (41) have presented data which show that the antagonism is strictly competitive. Also with *S. fecalis* the toxic effects of 'Aminopterin' were much more effectively blocked by citrovorum factor than by folic acid (41).

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### *L. Biological activity of the citrovorum factor*

In the light of the previous discussion, it is obvious that the citrovorum factor should be able to function biologically in lieu of folic acid. That this is the case has been established (41, 230). Although the new factor was reported by Hill and Briggs (159) to be fully as effective as folic acid when added to the diet of chicks, Broquist and his associates (42) found it to be somewhat less active when administered by this route, although equal in potency to folic acid when given parenterally. Presumably, the greater instability of the citrovorum factor accounts for its potentially lower activity, as compared with folic acid, when the two compounds are placed in the diet. The citrovorum factor can substitute for pteroylglutamic acid in folic acid-deficient rats rendered incapable of using folic acid by the administration of 25 μg. of 'Aminopterin' daily. Even though such animals were not protected by 5,000 μg. of folic acid daily (*v.s.*), the administration of from 25 to 250 μg. of citrovorum factor (leucovorin) not only gave full protection against the analogue, but initiated very rapid growth (230). Schoenbach and co-workers (274) have reported also that human subjects in whom there had developed marked evidence of the toxicity of 'Aminopterin' or 'A-methopterin' were fully relieved following the administration of citrovorum factor, despite the continued administration of the toxic analogue. This demonstration is of importance not only because of its practical significance, but also because it clarifies a point concerning which there was doubt (230), that is, whether the citrovorum factor could *reverse* as well as prevent the development of the toxic effects of the analogue. The *reversal* of toxicity observed by Schoenbach *et al.* (274) appears not to have been recognized by Burchenal and Babcock (45), who were unable to reverse the toxic effects of *massive* doses of 'A-methopterin' in mice with citrovorum factor, particularly if administration of the factor were delayed. Possibly the adverse results obtained in their mice are to be attributed in part to irreversible damage due to the previously injected analogue; however, it is possible also that with the relatively massive doses of analogue employed inadequate amounts of citrovorum factor were given. More recently, Burchenal and Kingsley-Pillers (47a) have reported that "in one patient who, on 2.5 mg. of 'Amethopterin' daily for eight days, developed toxic mouth ulcerations and leukopenia, doses of 45–60 mg. daily for twenty-one days were tolerated when covered by simultaneous administration of 3 mg. of citrovorum factor."

It has been reported that the citrovorum factor is active in pernicious anemia (83, 96, 168), and the activity exhibited appeared to be of an order of magnitude

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It has been reported that the citrovorum factor is active in pernicious anemia (83, 96, 168), and the activity exhibited appeared to be of an order of magnitude similar to that of folic acid. However, none of the data presented permits the conclusion that the two factors are of identical potency. Of pertinence, therefore, are the findings in a case[2] in which specially purified folic acid, 0.25 mg. daily by intramuscular injection, produced only a negligible hematopoietic effect during a ten-day period; however, immediately subsequent treatment with highly purified leucovorin, 0.25 mg. daily by the same route, caused a much more marked hematopoietic response. Administration thereafter of 4 mg. of leucovorin daily did not initiate a further increase in the number of reticulocytes, nor did daily injections of 4 mg. of folic acid. The erythrocyte count rose rapidly during the injections of the synthetic citrovorum factor. It must be concluded, therefore, at least in this case and by this method of administration, that the synthetic citrovorum factor was appreciably more active hematopoietically than was folic acid.

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[1] A crude mixture of compounds formed by reacting 2,5,6-triamino-4-hydroxypyrimidine with 2,3-dibromobutyraldehyde and *p*-aminobenzoylglutamic acid.

Actually, unless vitamin $B_{12}$ were involved, directly or indirectly, in the conversion of folic acid to the citrovorum factor, it would not be expected that the anti-pernicious anemia activity of the citrovorum factor should be much more than that of the parent compound. That no significant biochemical defect in the conversion characterizes the pernicious anemic state is indicated by the finding (44) that the urinary excretion of citrovorum factor activity by patients with pernicious anemia given folic acid is not significantly different from that of normal individuals.

On the other hand, the augmentation of the formation of citrovorum factor from folic acid which is caused by ascorbic acid (229, 366) suggests the possibility that profound disturbances in the availability of vitamin C might depress the conversion of folic acid to citrovorum factor and lead to the appearance of signs of deficiency of "folic acid." That such a disturbance in the metabolism of folic acid may occur has been convincingly demonstrated in scorbutic monkeys by May and associates (203, 204, 205). In these animals there develops very gradually a severe megaloblastic anemia which responds to a correction of the scorbutic state by treatment with vitamin C, or to folic acid in doses in excess of 750 μg. daily (without influencing the signs of scurvy). Since daily doses of 750 μg. of folic acid proved to be suboptimal in their hematologic effect, it is of considerable interest to note that synthetic (impure) folinic acid (citrovorum factor) in daily doses reported to be equivalent to only 7.5 μg. of pure material caused a prompt reticulocytosis followed by regeneration of the bone marrow to a normal cellular pattern, and improvement in intestinal function (diarrhea characterizes the folic acid-deficient state in this species). Thus it would appear that in the absence of adequate levels of ascorbic acid the "activation" of folic acid is performed inefficiently. This inadequacy can be circumvented (a) by administering excessive amounts of the precursor, (b) by giving minute amounts of the product of the conversion, or (c) by correcting the scurvy. In the absence of scurvy, it may be assumed that the minute amounts of folic acid (or citrovorum factor) obtained from intestinal bacterial synthesis, or otherwise, are adequate to supply the needs of the animal for the active compound.

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[2] We are grateful to Dr. Lee L. Conrad of the University of Oklahoma School of Medicine for permission to describe these unpublished findings, obtained with our collaboration.

similar to that of folic acid. However, none of the data presented permits the conclusion that the two factors are of identical potency. Of pertinence, therefore, are the findings in a case[2] in which specially purified folic acid, 0.25 mg. daily by intramuscular injection, produced only a negligible hematopoietic effect during a ten-day period; however, immediately subsequent treatment with highly purified leucovorin, 0.25 mg. daily by the same route, caused a much more marked hematopoietic response. Administration thereafter of 4 mg. of leucovorin daily did not initiate a further increase in the number of reticulocytes, nor did daily injections of 4 mg. of folic acid. The erythrocyte count rose rapidly during the injections of the synthetic citrovorum factor. It must be concluded, therefore, at least in this case and by this method of administration, that the synthetic citrovorum factor was appreciably more active hematopoietically than was folic acid.

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The significance of these studies to the megaloblastic anemia of human infants has been discussed appropriately by May and his associates (203). They comment that among the reported cases of megaloblastic anemia of infancy, 25 per cent were scorbutic, and in fully half of the cases the history disclosed a grossly inadequate intake of vitamin C. Significant also is the fact that a much shorter time is required to deplete the tissues of ascorbic acid than for signs of scurvy to develop (398). Attention is called also to a rather convincing item of circumstantial evidence, namely, the complete disappearance of megaloblastic anemia within the past few years among infants fed on proprietary artificial milks. Until about four years ago these foods were not supplemented with ascorbic acid and essentially none of the natural vitamin remained after the processing of the ingredients during manufacture of these preparations.

The metabolism of folic acid in adult humans with scurvy has been found to be abnormal (44, 113a, 366); thus, the urinary excretion of citrovorum factor, following daily doses of folic acid, was found to be very low as compared with that of normal subjects. It is of interest also that the ability to excrete the metabolite of folic acid did not increase promptly following the administration of ascorbic acid, as is the case with normal individuals. Conceivably, this delay in excreting the factor represents utilization of the material for storage in the tissues, although other factors may be involved.

### *M. Influence of folic acid on the metabolism of tyrosine*

Evidence that folic acid is in some manner concerned with the oxidative metabolism of tyrosine has been presented by Woodruff and associates (391, 392). They found that the disturbances of tyrosine metabolism characteristic of scorbutic albino guinea pigs could be largely corrected (marked reduction in the amount of "tyrosyl" compounds excreted in the urine) by the administration of folic acid. The vitamin was required in relatively large amounts, 5 mg. or more daily, or every other day. These observations suggest, in the light of the findings discussed previously, that the citrovorum factor is in some manner concerned with the catabolism of tyrosine. Some support for such a view was afforded by findings of Rodney and associates (259) and of Rienits (255a). On the other hand, in scorbutic monkeys the comparably abnormal metabolism of tyrosine is not corrected by folic acid (265) or by folinic acid-SF (266). In human infantile scurvy, folic acid, in doses of 15 mg. daily, was without favorable effect according to Woodruff (390); but Morris and associates (223) have reported that the expected tyrosyluria of scorbutic infants was inhibited by massive doses of folic acid (90 mg. daily). Work in our laboratories (227) has failed to demonstrate any significant influence of folic acid, citrovorum factor, or their antagonistic analogues, on the hepatic oxidation of tyrosine *in vitro*, either in the presence or absence of added $\alpha$-keto-glutarate (193). Also, Gaines, *et al.* could demonstrate no influence of folic acid-derived substances on the oxidation of tyrosine by rat liver *in vitro* (114a). However, the original observations of Woodruff and associates in guinea pigs, *in vivo*, have been confirmed (227). Whether the positive results obtained can be attributed to an interrelation with adrenal cortical function, in view of the effects obtained on the tyrosine metabolism of scorbutic

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## IV. THE CATALYTIC FUNCTIONS OF DERIVATIVES OF PTEROYLGLUTAMIC ACID AND OF COBALAMIN, AND THE FUNCTIONAL INTERRELATION BETWEEN THE TWO VITAMINS

It was planned originally to devote a considerable portion of this review on hematopoietic agents to a detailed consideration of those metabolic roles of folic acid and vitamin $B_{12}$ which have been disclosed by recent studies of bacterial, avian and mammalian organisms, tissues and extracts. It soon became evident, however, that this aspect of the subject already has grown to such size as to require more space than can be devoted to it in this review, except for a summarization in which only the most pertinent literature is discussed.[3]

### A. *Transfer of a "one-carbon unit"*

The actual catalytic roles of enzymes with which derivatives of folic acid and vitamin $B_{12}$ may be assumed to be associated have not yet been explained in any detail (with perhaps one exception), nor have such enzymes been isolated. Nevertheless, sufficient progress in our understanding of the biochemical functions of these vitamins has been made to permit a picture to be drawn, albeit a still somewhat hazy one. Perhaps the most notable recent advances toward the clarification of this picture have been the announcement of the structure of the citrovorum factor and its synthesis (leucovorin; folinic acid-SF) (37, 206, 247, 281) and the demonstration by Broquist and associates (38) of the citrovorum factor activity of 5,6,7,8-tetrahydropteroylglutamic acid. These findings give a rational structural basis for the concept that folic acid is concerned with the transfer of an active "one-carbon fragment." The "shuttling" of a formyl group, which the structure of these two molecules makes reasonable, indicates that the synthesis and degradation of many compounds essential to cell structure and function may require the participation of enzyme systems of which the citrovorum factors (or their derivatives) are components. Actually, considerable evidence for participation in this manner of folic acid-derived compounds in biological syntheses and degradations has been available for some time. Such reactions appear to be concerned with the formation of a pyrimidine (thymine) and its derivative (thymidine); purine derivatives (*e.g.*, inosine-5-phosphate *vs.* the ribotide of 4-amino-5-imidazdecarboxamide); the utilization of glycine in a variety of reactions, in particular for the formation of a "one-carbon fragment"; and in the synthesis of porphyrins.

Perhaps the earliest clue to a function of folic acid was the finding that the growth of *S. fecalis*, and certain other lactobacilli, can be supported not only by minute amounts of folic acid, but also by thymine in concentrations at least 5,000 times greater (296, 310, 313). It was suggested (310) that folic acid might function as a coenzyme in the synthesis of thymine and thus of nucleic acid. Subsequent developments have supported these views and they will receive additional attention, since they are very pertinent to the problems posed by the hematopoietic action of folic acid.

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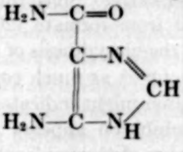
[3] An excellent review, "The role of vitamin $B_{12}$ in metabolic processes," by Jukes and Stokstad (175), gives consideration to folic acid as well. Also, a review by one of us (A.D.W.) in collaboration with C. A. Nichol, "Water-soluble vitamins concerned with one- and two-carbon intermediates," will devote attention to the participation of folic acid and vitamin $B_{12}$ in metabolic processes; this should appear in the Annual Review of Biochemistry (1952).

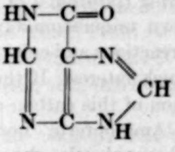
In the meantime, however, more can be gained from a brief review of the mechanisms of purine synthesis, since notable advances recently have occurred in this field.

### *B. Synthesis of purines*

1. *Imidazolecarboxamide.* It will be recalled that when the growth of cultures of *E. coli* is partially inhibited by a sulfonamide a diazotizable amine accumulates (104). This compound was isolated and studied by Stetten and Fox (309) and identified as 4-amino-5-imidazolecarboxamide by Shive and his associates (280), who recognized its precursory relationship to the purines.



*4-Amino-5-imidazolecarboxamide*



*Hypoxanthine*

The compound accumulates also in cultures of *E. coli* inhibited by 'Aminopterin' (394) and of a purine-requiring mutant of this organism (125). These findings indicate that the sulfonamides, by interfering with the synthesis of folic acid, and 'Aminopterin' by preventing its conversion to citrovorum factor and by competing with the latter, limit the rate of introduction of a "one-carbon fragment" needed for the conversion of the imidazole to a purine. However, the recent work of Greenberg (127) has shown that this ring-closure, at least in a pigeon liver system, apparently does not occur with the free imidazole derivative. The latter represents a degradation product of the original intermediate, which is a ribotide of this compound. This ribotide, by accepting the "one-carbon fragment," presumably a formyl group, is converted into inosine-5-phosphate (inosinic acid), the purine component of which is hypoxanthine. Greenberg has shown further (128) that for the optimal synthesis of inosine-5-phosphate, *de novo*, the following compounds are necessary: glycine, bicarbonate, formate, glutamic acid or glutamine, a source of ribose-1-phosphate, biotin and citrovorum factor; also, a coupled source of energy is required. It is of particular interest, in connection with the role of citrovorum factor, that either formaldehyde or formate can serve as the donor of the "one-carbon fragment"; neither is converted to the other, but presumably each to a common intermediate.

2. *Inosinic transformylase.* Buchanan (43) has shown that incubation of formate with inosinate in a pigeon liver system similar to that employed by Greenberg results in a rapid exchange of the carbon in position-2 of the hypo-

xanthine moiety with the carbon of the formic acid. Having shown that citrovorum factor catalyzes this exchange reaction, the authors have termed the enzyme system involved *inosinic transformylase;* this represents the first partially purified enzyme system in which a function of the citrovorum factor can be rather clearly recognized. It will be appreciated, of course, that the function of the citrovorum factor in this carbon-transfer, potentially at least, is as much concerned with purine degradation as purine synthesis.

3. *Other aspects of purine synthesis.* Very recent findings (16) with a mutant of *E. coli* indicate that in the conversion of the aminoimidazolecarboxamide to purine, vitamin $B_{12}$ also plays an essential role. In view of the evidence (*v.i.*) that this vitamin is concerned in some manner with the formation of desoxyribosides of purines and pyrimidines, it may be that for the necessary conversion of a precursor of the imidazole derivative to a nucleotide (in this case presumably a ribotide), vitamin $B_{12}$ is required; however, many other sites of participation of this vitamin are possible.

Still unclarified is the mechanism of introduction of the carbon in position-8 of the purine ring (position-2 of the imidazole derivative). Since this carbon also has been shown unquestionably to be derived from formate (201, 299), the nature of the reactions and cofactors involved in the biosynthesis of the imidazole ring are of much interest. If the citrovorum factor is as much concerned with the introduction of this carbon atom as speculation might indicate, it is curious that neither 'Aminopterin' nor sulfonamide inhibition appears to block the synthesis of the imidazole, although the conversion of the imidazole to a purine is inhibited. Possibly the interference will be explained on a mass action basis, but, as yet no one has isolated an appropriate precursor of aminoimidazolecarboxamide.

That the citrovorum factor plays a similar role in the synthesis of purines by mammals, as has been demonstrated with *E. coli* and avian species (*v.s.*), is indicated by the studies of Skipper *et al.* (291). These workers showed that the ability of mice to incorporate formate (labeled with $C^{14}$) into the purines of nucleic acid is profoundly depressed by the administration of 'Aminopterin.'

### C. *Synthesis and hematopoietic activity of uracil and thymine*

In the synthesis of thymine, concerning which some mention already has been made, the elucidation of biochemical detail which has been advanced is not comparable to that attained with the purines. Extensive studies with bacteria (175, 296, 310, 313) have shown, however, that folic acid is not only involved in the synthesis of thymine, but in some cases it seems to be necessary for the conversion of thymine to thymidine. Although it is logical to assume that the citrovorum factor transfers a formyl group to the carbon in position-5 of uracil, possibly to form a 5-hydroxymethyluracil which is subsequently reduced to 5-methyluracil (thymine), no concrete evidence for such a pathway of conversion has been advanced. Most pertinent perhaps was the demonstration (137) that administration to rats of uracil-riboside or cytosine-riboside labeled with $N^{15}$, caused the appearance in desoxyribonucleic acid of labeled thymine and cytosine.

These observations suggest not only that "the rat is able to form desoxyribose from ribose when the sugar is bound to cytosine," but also that the unmethylated pyrimidine ring can be methylated in the body.

The observation of Vilter et al. (350) that the administration of uracil in daily doses of 15 to 30 *grams* to patients with pernicious anemia often causes an hematologic remission is of great interest. Thymine, in daily doses of 15 grams, was found by these workers, in confirmation of Spies and associates (300), who had used somewhat smaller doses, to be capable of inducing a similar hematologic response. Vilter and associates postulate that uracil serves as a precursor of thymine, which in turn is utilized for the synthesis of nucleic acid. From the size of the doses required with these pyrimidines, it is evident that utilization must be extremely inefficient, at least by individuals whose supplies of vitamin $B_{12}$ and functional derivatives of folic acid are minimal. Actually, nothing is known concerning the degree to which these two very poorly soluble compounds are absorbed by *man* or what proportion of the amount absorbed may be used for nucleic acid synthesis and what proportion may be excreted or degraded. Studies of absorption and excretion could be done with the aid of microbial assays and actual utilization, as well as excretion and degradation, could be followed with isotopically labeled materials. In *rats* it has been shown that $N^{15}$-labeled free pyrimidines are *not* utilized for nucleic acid synthesis, although moderate amounts can be absorbed and catabolized (246a).

One of the obstacles to ready acceptance of the concept that thymine functions hematopoietically in pernicious anemia by supplying the principal compound needed (that ordinarily is supplied by a reaction catalyzed by derivatives of folic acid), has been thought to be the failure of thymine favorably to influence folic acid-deficiency in rats and chicks (174). This failure might be considered to represent the inability of a *single product* of many reactions in which citrovorum factor is involved to relieve the *multiple* needs for the vitamin in severely deficient animal species. However, as stated previously, adult rats cannot utilize free thymine for nucleic acid synthesis (246a), although thymidine enters desoxyribonucleic acid in this species (250a). In view of the fact that patients with pernicious anemia in relapse respond hematopoietically to massive dosage with free uracil or thymine it may be that man differs from the rat with respect to the utilization of free pyrimidines. This view requires, of course, that the hematopoietic inadequacy of pernicious anemia be regarded primarily as a need for compounds derived from thymine. Why this should be the case is difficult to explain unless the reactions involved in the synthesis of thymine derivatives by man are the most "sensitive" of those requiring derivatives of folic acid and vitamin $B_{12}$.

Considerable support for the view that thymine derivatives are particularly concerned with hematopoiesis is to be found in our observation (360) that pigs with the macrocytic anemia and megaloblastosis of folic acid deficiency have responded hematopoietically to thymine (in doses of 10 grams daily). However, there is no assurance that thymine in these cases was utilized for synthetic purposes; for example, it could have had a xanthopterin-like action (148). Again, the need for studies of labeled thymine is obvious.

That thymine should prove effective in patients with pernicious anemia *refractory to folic acid* might seem quite unreasonable, if thymine (or a closely related compound) is to be regarded as a product of a reaction in which derivatives of folic acid participate. Yet, the observations of Vilter and associates (350) on the effectiveness of thymine were made under precisely such conditions; hematologic relapse had occurred even though folic acid had been continued for months in doses of 30 mg. three times weekly. The most reasonable explanation of this failure of folic acid to induce hematopoiesis would appear to be that folic acid became ineffective when the deficiency of vitamin $B_{12}$ became sufficiently profound. We have suggested previously (148) that hematopoiesis is inhibited by a deficiency of either vitamin, if the deficiency is sufficiently severe. It might be suggested that under the conditions described by Vilter and associates the amount of "thymine" biologically synthesized, and the proportion of this that was utilized, were inadequate for the formation of erythrocytes at a rate equal to their degradation. On the other hand, massive doses of thymine still could cause a response, despite the presumed deficiency of vitamin $B_{12}$; however, if the latter deficiency became sufficiently severe, thymine, according to these views, should become ineffective because of the need for other compounds with the synthesis of which this vitamin must be concerned.

It should be emphasized that these concepts are speculative, but they may have some utility as working hypotheses concerning the action of these hematopoietic agents. Also, comment should be made concerning the use of the term "thymine"; presumably, the compound synthesized in the tissues as a precursor of thymidine is not thymine itself but a related compound, as has been suggested by others (Lampen and Jones, 191). A not unlikely sequence of events is as follows: aspartic acid, ureidosuccinic acid (394a), orotic acid (394a), uridine, cytidine, desoxycytidine (137), desoxy-5-methylcytidine, thymidine, or variations of such a pathway of events.

### *D. Relationships among hematopoietic factors in swine*

Speculations of the above type are compatible with observations made in swine with deficiencies of either folic acid or vitamin $B_{12}$, or both. Studies in such animals showed that both folic acid and vitamin $B_{12}$ are necessary for normal hematopoiesis. Swine made deficient by maintenance on a purified diet extremely low in folic acid, with added succinylsulfathiazole and a crude folic acid antagonist (x-methylfolic acid, 110) develop severe macrocytic anemia with megaloblastic hyperplasia of the bone marrow which responds rapidly and completely to the administration of folic acid (55, 147). One folic acid-deficient animal was given large amounts of "refined" liver extract (15 U.S.P. units per ml.), containing no significant amount of folic acid derivatives, during a period of nearly a year (148). Progressive macrocytosis and marrow megaloblastosis developed, but the animal did not die, and was able to maintain a stable, though quite abnormal, hematopoietic state. This result may have been due to the availability of minute amounts of folic acid from some undefined source (possibly bacterial synthesis in the intestinal tract was not blocked completely). In any case, the administration of folic acid caused prompt restoration of normal blood values with disappearance of macrocytosis.

Vitamin $B_{12}$-deficient pigs, on the other hand, developed severe anemia of a *normocytic* type, not associated with megaloblastic hyperplasia of the marrow and this anemia responded readily to the administration of cyano-cobalamin (148). Very extensive studies with swine by Cartwright, Wintrobe, and their associates (54, 55, 56, 386), have led to similar observations and conclusions.

If these observations apply to human pernicious anemia, as would appear reasonable, the macrocytosis and megaloblastosis of that disease must be regarded as a reflection of a profound need for compounds derived from folic acid. Since the basic deficiency in pernicious anemia must be recognized as due to a failure to utilize vitamin $B_{12}$, the explanation for the disturbance in folic acid metabolism greatly needs to be explained. This will be considered subsequently. First, however, additional remarks should be made concerning other biochemical roles of vitamin $B_{12}$ and folic acid.

### *E. Role of thymidine*

Mention has been made of the fact that the metabolic requirements of various bacteria for vitamin $B_{12}$ can be obviated by addition to the media of the desoxyriboside of thymine (thymidine). In some species (*e.g.*, *L. leichmannii*, ATCC 7830), good growth can be obtained in the absence of thymidine and vitamin $B_{12}$ with a desoxyriboside either of purines or of pyrimidines other than thymine, but only when folic acid is supplied. In view of convincing evidence (207) that certain enzymes are concerned with the utilization of the desoxyribose moiety of one nucleoside for the synthesis of another, this situation offers no great complications. But, as yet, no evidence has been offered to account for a function of vitamin $B_{12}$ in reactions concerned with the synthesis of thymidine, any of the other nucleosides containing desoxyribose, or desoxyribose itself. However, that vitamin $B_{12}$ is involved in the conversion of a thymidine-precursor to thymidine seems to be an excellent possibility, at least in the vitamin $B_{12}$-requiring lactobacilli.

In view of the hematologic response of patients with pernicious anemia to thymine (in massive doses) a question naturally arises as to the effect of thymidine. Unfortunately, this compound is quite difficult to prepare in large quantities and only a little work with it in human subjects has been reported. Reisner and West (253) state that only a slight reticulocytosis (and no improvement in total erythrocyte count) followed the injection, in three patients, of from 5 to 150 mg. of the desoxyriboside; and Ungley (343) obtained no response to a dose of 48 mg. However, Hausmann (141) has reported that total doses of thymidine of 2.0 and 2.8 grams, respectively, in two cases of pernicious anemia in relapse "exerted a blood-forming action comparable to that of corresponding doses (*sic*) of vitamin $B_{12}$, folic acid, or thymine." Since the thymidine used in these studies of Hausmann was prepared from a liver extract, the question of its possible contamination with vitamin $B_{12}$ must be raised; however, the author claims to have excluded this possibility. That such doses of the soluble compound, thymidine, should be effective in inducing hematopoiesis in pernicious anemia is not too surprising, in the light of the responses to the very much less soluble precursor, thymine.

Among the various mutants of *E. coli* which have been developed, it is indeed curious that none of those which respond to vitamin $B_{12}$ is able to utilize thymidine or other nucleosides in lieu of the vitamin (87). However, that thymidine, as might be expected, is of metabolic significance to *E. coli* was indicated by experiments with 'Aminopterin' (111). High concentrations of the antagonist inhibited growth of the organism despite the fact that folic acid is not required by *E. coli*. Although this inhibition was not affected by thymine, thymidine restored growth to an essentially normal level. These findings would suggest that thymidine is synthesized by *E. coli* from precursors other than thymine and that the citrovorum factor is involved in the process.

### *F. Synthesis of methionine and the labile methyl group*

In the mutants of *E. coli* the need for vitamin $B_{12}$ has appeared to be related particularly to the synthesis of methionine (Davis, 85; Davis and Mingioli, 87). It was concluded that vitamin $B_{12}$ is concerned with the methylation of homocysteine. The nature of the slow growth on vitamin $B_{12}$ of two of the mutants (which grow rapidly on methionine) has been suggested as possibly involving slow conversion of the vitamin to a coenzyme of methylation.

These findings in bacterial metabolism have their counterpart in metabolic studies in animals (reviewed in detail by Jukes and Stokstad (175) and by Emerson and Folkers (100)); such studies have shown incontrovertibly that vitamin $B_{12}$ is involved in the synthesis of methionine. However, it is not yet entirely clear whether the vitamin is concerned with the synthesis, *de novo*, of the labile methyl group, or with its transfer to a precursor of methionine (presumably homocysteine or a derivative of it), or with both processes. In any case, the labile methyl group, once it has become a part of methionine can be transferred readily to a variety of compounds for which methionine appears to be the specific donor of a methyl group. However, it has been shown (52, 53) that, before methionine can give up its potentially-labile methyl group, an enzyme-catalyzed "activation" reaction must occur. This reaction requires a source of energy, *e.g.*, adenosine triphosphate, and a sulfhydryl compound such as glutathione. The altered methionine, the structure of which has not yet been established, is the actual methyl donor. With the aid of specific enzymes, a methyl group then can be transferred to the precursors of such compounds as creatine and N-methylnicotinamide; very probably epinephrine acquires its methyl group from the same compound. In none of these reactions concerned with the "activation" of methionine has evidence been found for a participation of vitamin $B_{12}$ or the citrovorum factor and it is likely that these cofactors participate at an earlier stage.

Although it had been thought that the labile methyl group is obtained exclusively from exogenous sources such as choline, betaine and methionine, it has now been established that the animal organism can synthesize this one-carbon radical, although the rate of such synthesis may not be commensurate with optimal performance. It was shown by Sakami (263) that the carbon of $C^{14}$-labeled methyl groups of administered-acetone appear in the methyl carbons of tissue choline and methionine. Suspecting that this indicated an intermediary conversion of the acetone-methyl to a formate-like compound, rather than a direct transfer, Sakami and Welch (264) studied the utilization of $C^{14}$-formate by rats, and by slices of surviving rat liver *in vitro*. It was proved that formic acid can be converted by hepatic enzyme systems into the methyl groups of methionine and choline, and preliminary evidence that folic acid is involved in the conversion was presented. That folic acid could be involved in the synthesis of methionine had been suggested by the studies of Bennett in 1949; she had shown (17) that folic acid is needed for growth when homocystine is fed to rats in place of methionine, and had suggested that vitamin $B_{12}$ also could be involved in methionine synthesis.

The findings with formate have been extended by duVigneaud and associates (92), who found that formaldehyde and methanol, as well as formate, can be used by intact rats for methyl synthesis; also studies by other investigators (7a, 170, 308, 355) have confirmed and extended these observations on methyl synthesis. By using germ-free rats, duVigneaud and associates (92) showed, as had the earlier liver slice experiments, that the synthesis of the labile methyl group can be performed by enzymes of the rat, in addition to that presumably carried out by symbiotic intestinal bacteria.

That vitamin $B_{12}$, as well as folic acid, in some manner promotes the synthesis of methionine by animals as well as by bacteria was clearly indicated by various studies, as has been mentioned earlier. In this respect, the experiments of Stekol and Weiss (306) were suggestive. They found that *rats* on an amino acid diet containing homocystine in place of cystine and methionine were unable to grow without vitamin $B_{12}$; after a lag period of two weeks, loss of weight and death occurred if the vitamin was not given. These findings suggested that vitamin $B_{12}$ is required for the conversion of homocystine to methionine. Experiments of Jukes and his associates (176) in *chicks* also pointed to a very similar locus of action of vitamin $B_{12}$. These workers showed that chicks given a diet deficient in both vitamin $B_{12}$ and methionine, but containing folic acid, failed to grow when given homocystine, even though betaine (as a methyl donor) and dimethylaminoethanol (for choline synthesis) were fed. Although growth occurred when methionine was added to the diet (without vitamin $B_{12}$), homocystine, or homocystine plus betaine, promoted growth only when vitamin $B_{12}$ was made available. Since deuterium-labeled methyl groups of betaine have been shown to be biologically transferable to choline and creatine, presumably by way of methionine (93), these results point strongly to a participation of vitamin $B_{12}$, either directly or indirectly, in the transfer of a labile methyl group from betaine (or some other intermediate methyl donor) to a precursor of methionine. Since growth also occurred with homocystine and vitamin $B_{12}$, in the absence of dietary betaine, it may be concluded that under these conditions methionine was synthesized from homocystine and one-carbon metabolic intermediates. From similar studies of Schaefer, *et al.* (270a) in chicks deficient in vitamin $B_{12}$ it could be concluded that this vitamin is involved in the utilization of either betaine or methionine for the synthesis of choline from methylaminoethanol, while aminoethanol could not be used for the synthesis of choline, even though these methyl donors were supplied (171a; 171b), together with vitamin $B_{12}$ (270a). These findings suggest the possibility that not only is vitamin $B_{12}$ involved in transmethylation from betaine but also from methionine.

The knowledge concerning the structure of cyano-cobalamin is insufficient to give a clue to a mechanism of the participation of the vitamin in the transfer of methyl groups. It must be emphasized that the role of vitamin $B_{12}$ may be indirect; that is, it may be related to the synthesis of another compound which is more directly concerned with the actual transfer of the labile methyl group. Evidence that this is the case may be found in the interesting report of Oginsky (235), who demonstrated that the hepatic tissue of vitamin $B_{12}$-deficient rats formed *in vitro* less methionine from homocystine and either choline or betaine than did that of animals treated with the vitamin. However, for recovery of the synthetic ability, days rather than hours following treatment with the vitamin were required. This result may be contrasted with that of Sakami and Welch (264) who demonstrated a prompt response (within 2½ hours) following the injection of folic acid in deficient rats, in which a reduced ability to convert $C^{14}$-formate to the methyl group of methionine had been found.

The demonstration of the lability of the cyano group of vitamin $B_{12}$ (35, 180) is most suggestive of a possible function of the vitamin (33, 308). Through the transfer of the cyano group, vitamin $B_{12}$ could be considered, like the citrovorum factor, as involved in the "shuttling" of a moiety containing only one carbon atom. Conceivably, the transfer of the intact cyano group might be involved in the synthesis of nucleosides, but if so, no evidence for such a remarkable participation has been advanced. Also this speculation, of course, offers no clue to a function of cyano-cobalamin in the transfer of labile methyl groups to a derivative of homocystine.

### *G. Formation of sulfhydryl groups*

Dubnoff has suggested, on the basis of studies with isolated liver systems (90) and with mutant strains of *E. coli* (91), that vitamin $B_{12}$ is involved, directly or indirectly, in reactions which lead to the formation of sulfhydryl (—SH)—containing compounds from their disulfide precursors (—S—S—). Thus, he postulated that vitamin $B_{12}$ is concerned with the reduction of homocystine to homocysteine and that it is this function which enables the vitamin to participate in methyl-transfer. In other words, it is the preparation of the recipient compound for acceptance of a methyl, rather than involvement in the actual transfer of a methyl, that he would consider as dependent in some way upon vitamin $B_{12}$. There are many attractive aspects to this theory. If it could be applied generally to —S—S—$\rightleftharpoons$—SH equilibria, it is obvious that the function of the vitamin could be regarded as of the most basic significance. As is well known, a very large proportion of enzymes contain free —SH groups, without which their catalytic functions cannot be exerted. Among such enzymes are those concerned with the "activation" of methionine (53) and the cleavage of folic acid from its polyglutamates (215). Although the findings of Dubnoff have not been confirmed, the reports of further studies in this intriguing field are awaited with interest.

### *H. The "formate pool"*

Regardless of the role ultimately assigned to vitamin $B_{12}$ in the handling of the methyl group, there is no longer any reason to doubt the participation of derivatives of folic acid in the formation of this labile radical. That folic acid is involved in the utilization of "formate" for this and other purposes and in the metabolism of compounds which contribute to the "formate pool" appears to have been established clearly (43, 98, 346, 264, 336, 337). Contributions to the "formate pool" may be made not only by formate, formaldehyde and methanol, but also by glycine ($\alpha$-carbon) 262, 355), serine ($\beta$-carbon) (97, 98, 170, 355), threonine (208, 250, 279), purines (carbons 2 and 8) (43, 274a), the methyl groups of choline, betaine and methionine (137a, 199, 286), and undoubtedly by many other materials. Presumably, the acquisition of a one-carbon unit from these molecules or derivatives of them is the task of an enzyme or group of enzymes containing tetrahydropteroylglutamic acid or a derivative of it. In turn, enzymes containing a similar prosthetic group may be assumed to operate in the uptake of "formate" from the pool for synthesis of some of these compounds (not glycine and threonine) and others yet to be disclosed.

One might suggest speculatively that the citrovorum factor in its functional form, might well be in combination not only with a protein (apoenzyme), but also with a pentose in position-8. This formulation would agree remarkably well with that of each of a long list of biologically important heterocyclic compounds, *e.g.*, riboflavin, adenosine (and other nucleosides), and the moiety of vitamin $B_{12}$ demonstrated to contain ribose (100).

### *I. Labile methyl groups and hematopoiesis*

Attempts to demonstrate an hematologic effect in pernicious anemia of compounds containing either labile, or potentially labile, methyl groups have been made. Vilter and associates (350) showed, in a pernicious anemic patient with an erythrocyte count of about 2,000,000 per cmm. of blood who had failed to respond to uracil (30 grams daily), that the co-administration of choline chloride, 3 grams, and methionine, 6 grams, daily for ten days, caused a reticulocytosis of 7.4 per cent on the ninth day, and a significant rise in the red blood cell count. Further, the patient experienced considerable improvement in appetite and sense of well-being. It is not possible definitely to attribute this result to a direct transfer of the labile methyl group to uracil for the synthesis of thymine, as was suggested by the authors (350), although there are no data to exclude this possibility. Equally reasonable, however, might be that these methyl donors greatly augmented the size of the "formate pool" from which, with the aid of traces of catalysts derived from folic acid and vitamin $B_{12}$, derivatives of thymine and many other compounds could be synthesized.

### *J. Relationships between vitamin $B_{12}$ and derivatives of folic acid*

From all that has been said concerning the extremely important reactions with which folic acid and vitamin $B_{12}$ are concerned in bacteria and in chicks and rats, and from the evidence which Cartwright and associates (55) and our laboratories (147, 148) have presented from studies of hematopoiesis in swine, it should be clear that each of these substances is essential for several reactions encountered in cellular metabolism. Because of the magnitude of the task of manufacturing enormous numbers of cells, the first indication of a deficiency of the one or the other of these substances may be revealed by the blood-forming organs.

However, with this basic and most fundamental point established, we are still faced with the need for explanations of (1) the ability of each of these two vitamins to induce an hematopoietic response in pernicious anemia and (2) the deficiency of functional forms of folic acid in that disease, which must be recognized primarily as due to a deficiency of vitamin $B_{12}$. Clearly, a role of vitamin $B_{12}$ in the metabolism of folic acid is indicated, but as yet none has been established as occurring uniformly.

1. *Utilization of conjugated forms of folic acid.* In 1946 two independent groups of investigators, one in Ann Arbor and the other in Cleveland (22, 144, 329, 361, 362), described studies, using a total of eight patients with pernicious anemia in relapse, which appeared to offer a clue to the secondary deficiency of folic acid in this disease. Each group found that the conjugated form of folic acid known as "heptaglutamate" was ineffective in the doses used, whether given by mouth or parenterally, either in inducing an hematologic response in pernicious anemic subjects or in causing the urinary excretion of free folic acid that followed the administration of this compound to *normal* human subjects. In these patients the molar equivalent dosages of pteroylglutamic acid produced the usual hematopoietic effects and administration was followed by the usual urinary excretion of substances active for *L. casei* and *S. fecalis.*

These findings were interpreted as signifying that a deficiency of the "antipernicious anemia factor" (vitamin $B_{12}$) led to a failure in the utilization of the conjugated form of folic acid. Since by far the larger proportion of the folic acid normally ingested appears to be in a conjugated form, this finding apparently accounted for the development of a deficiency of folic acid in pernicious anemia. However, patients with pernicious anemia have been found subsequently in whom conjugated materials have proved effective hematologically (58, 277, 360, 381).

Bethell and associates (22, 329) have shown that patients with pernicious anemia are more susceptible to inhibitors (apparently nucleic acids) of the enzymes (conjugases) that release folic acid from the conjugated form than are normal individuals, whereas patients treated adequately with liver extract are not abnormally susceptible to these inhibitors. Whether this defect is a direct result of the deficiency of vitamin $B_{12}$ is uncertain. However, administration of potent liver extracts did not immediately increase the urinary output of pteroylglutamic acid in untreated pernicious anemia patients. In two cases of tropical sprue, one of whom was given daily doses of pteroylhexaglutamylglutamic acid and the other "fermentation folic acid," or pteroyl-di-γ-glutamylglutamic acid, the addition of liver extract by intramuscular injection increased within 24 hours the urinary output of substances active for *L. casei* and *S. fecalis* (326).

Sharp and Vonder Heide (277) also observed a remarkable increase in the urinary excretion of "folic acid" in a patient with pernicious anemia given the conjugate, subsequent to the daily administration of powdered stomach ('Ventriculin'). After a rather erratic daily excretion of free "folic acid" while 6 mg. of the conjugate was given daily, the elimination fell to less than 4 μg. of "folic acid" per day; however, fifteen days after the daily administration of 'Ventriculin' (30 grams) was begun, the urinary excretion of "folic acid" was increased suddenly to over 3,000 μg. daily.

After study of the metabolism of pteroyl-di-γ-glutamyl-glutamic acid or "fermentation folic acid," as well as the synthetic compound, pteroyl-α-glutamylglutamic acid, Wilkinson and Israëls (381) concluded that "patients with pernicious anemia can hydrolyse and utilise folic acid conjugates. . . ." However, this conclusion was severely criticized by Stanley Davidson (84) who pointed out that the amounts of these pure compounds used by these workers greatly exceeded those which would be present in the natural diet, which also appears to contain inhibitors of the enzymes which cleave the conjugates of folic acid. Also, it should be appreciated that the ability freely to hydrolyze the "triglutamate," or an "abnormal diglutamate," offers no assurance that the more widely distributed "heptaglutamate" will be as amenable to cleavage. Davidson shrewdly points out, "If a patient with pernicious anaemia were able to obtain 1 or 2 mg. of free folic acid daily from his food, he would not develop a megaloblastic anaemia. . . . Vitamin $B_{12}$ may act by facilitating the release of folic acid from the conjugates present in natural foods, or, alternatively, it may promote their absorption and utilisation in some manner as yet not determined."

The difficulties of drawing a really valid conclusion concerning the utilization of the conjugated forms of folic acid are obvious. It would appear that although inefficient cleavage of the conjugated derivatives to form free folic acid *may* be a direct result of a deficiency of vitamin $B_{12}$, it is more likely to represent a secondary manifestation of the deficiency of this vitamin, and this biochemical defect, in a severe form, may or may not be present in any single case. On the other hand many of the studies of conjugated folic acids have not been done with "heptaglutamate" in amounts equivalent to only 1 or 2 mg. of free folic acid daily. Certainly, when small doses of "heptaglutamate" were given, as in the studies of the Ann Arbor and our groups, profoundly abnormal utilization was encountered in a very significant number of cases of pernicious anemia. The subject deserves additional investigation.

2. *Metabolic handling of folic acid derivatives in pernicious anemia.* It has been mentioned previously that several patients with pernicious anemia studied by Burchenal (44) were able effectively to convert free folic acid (in doses of 10 mg. daily) to the citrovorum factor, as evidenced by the urinary excretion of material with activity for *Leuconostoc citrovorum*. However, no information is

available as yet concerning the ability of such individuals comparably to metabolize small doses of the "heptaglutamate." Such a study might prove of interest, since both cleavage of the conjugate and metabolic alteration to the citrovorum factor would be involved.

No information is available concerning the metabolic conversions of folic acid derivatives subsequent to the stage characterized by the structure assigned to leucovorin (folinic acid-SF). The possibility that vitamin $B_{12}$ is concerned in some manner, either directly or indirectly, with a later reaction that may occur prior to the formation of the active cofactor-apoenzyme complex cannot be overlooked. Thus, if the functional form of the citrovorum factor should prove to be a nucleoside comparable in structure to so many other cofactors, particularly if it were a desoxyriboside, an opportunity for a deficiency of vitamin $B_{12}$ to depress its synthesis conceivably would be afforded.

Actually, there is an almost incredible hiatus in our knowledge of the nature and amount of the derivatives of folic acid present in the tissues (particularly the liver) of patients with pernicious anemia in relapse, or after treatment with vitamin $B_{12}$. Nor is much known concerning the capacity of the tissues of such individuals enzymatically to hydrolyze conjugated forms of folic acid, or to carry out various syntheses with the catalysis of which derivatives of citrovorum factor and vitamin $B_{12}$ are concerned. The reason for our ignorance in these respects is fairly obvious: one does not lightly consider the removal of significant amounts of liver or other tissues, for analytical and enzyme activity studies, from patients with severe, untreated pernicious anemia. Although such studies undoubtedly will be conducted, apparently none has been described as yet. In the light of recent studies of radioactively labeled glycine ($-2-C^{14}$) in man (20), information soon should be obtainable concerning the metabolism of this compound (with which folic acid is certainly involved) in patients with untreated pernicious anemia, as compared with their subsequent behavior following treatment with either folic acid or vitamin $B_{12}$.

Brief comment should be made concerning the well-established fact that for arresting neuropathologic changes in those portions of the nervous system prone to degenerate in severe pernicious anemia, not only is folic acid of no value, but also there is strong evidence to suggest that treatment with folic acid alone may be harmful. Several investigators (136a, 145, 261, 352, 380) have observed sudden and rapidly progressive neurologic relapse in folic acid-treated patients with pernicious anemia. It has been suggested (145) that the mechanism of this phenomenon involves the aggravation of one deficiency (of vitamin $B_{12}$) by vigorous treatment of a co-existing deficiency (of folic acid). Claims that folic acid is in itself toxic for nerve cells and may function as a metabolic antagonist for glutamic acid (261) have been refuted (68a, 109a, 140).

In conclusion, it can be stated unequivocally that many years of clinical experience have proved that "refined" liver extracts, containing essentially no folic acid or citrovorum factor, almost invariably are capable of producing and maintaining a normal hematologic and neural status in patients with pernicious anemia. All available evidence indicates that crystalline vitamin $B_{12}$ is similarly effective. Consequently, it would appear that in pernicious anemia any defect in the metabolic handling of folic acid and its derivatives is corrected by the administration of adequate amounts of vitamin $B_{12}$. The extensive investigations of the metabolic roles of folic acid-citrovorum factor and of the cobalamins, which are now in progress, offer promise that the biochemical functions and interrelationships of these two groups of essential factors soon may be clarified.

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# SOME BASIC ASPECTS OF THE PHARMACOLOGY OF SYNTHETIC CURARIFORM DRUGS

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## INTRODUCTION

The preparation of crystalline *d*-tubocurarine, the elucidation of its structure, its introduction into clinical medicine and the decisive demonstration of its value as a therapeutic agent have resulted in considerable effort on the part of organic chemists to prepare other drugs with a similar action. Prior, however, to the widespread clinical use of *d*-tubocurarine, pharmacologists have, since the work of Crum Brown and Fraser, been interested in the action of onium ions of various types at the neuromuscular junction.

The review published in 1936 by Ing (77) contains a comprehensive and critical account of this older fundamental work. Since 1936, books by MacIntyre (93), Bovet and Bovet-Nitti (13) and Henry (64) have appeared. Also, reviews by Bovet and Bovet-Nitti (14, 15), Craig (43), Paton (104), Paris (103), Robson and Keele (115), Fourneau and Janot (55) and Marsh and Herring (94) have been published. These reviews cover the literature pertaining to curare and synthetic substitutes in a detailed and comprehensive manner. No attempt will be made in this article to repeat this material. Instead, attention will be drawn to progress in other fields, which, in the opinion of the author, bears on the problems encountered, and an attempt will be made to integrate and assess this information. The task is difficult for many reasons. The multiplicity and the complexity of structural factors required for high curariform activity prevent their individual isolation and analysis. Moreover, the study of the influence of altered chemical structure on molecular properties gives rise to problems in physical chemistry which have not yet been solved. On the biological side, lack of information as to exactly how curare exerts its action renders an unequivocal definition of curariform action impossible.

In this article an attempt has been made to analyze some of the problems encountered by resolving them into their fundamental components. One of the main objects of this method is to render the gaps and deficiencies in our knowledge of the subject more obvious.

### *Mode of Action of Curare*

A provisional definition and description of curariform action are desirable for several reasons. Since there are different ways in which the transmission of excitation from nerve to muscle can be stopped, drug structure cannot be related to crude blocking action. Moreover, when new drugs are assayed for curariform action, it is important to establish that they do not block neuromuscular transmission in some other way. Finally, all available knowledge of the reaction in which the drug participates will contribute to our insight into the structure required for optimal activity.

Since little is known of the mode of action of curare on the molecular level, we will have to be satisfied to use those experimental characteristics of the action of *d*-tubocurarine, which seem to be associated with its basic action, as a definition of the term curariform. It is generally believed that the reaction between curariform drugs and tissues can be represented by the completely reversible reaction:

$$\text{Curare} + \text{Receptors} \rightleftharpoons \text{Curare-Receptor Complex} \qquad (1)$$

This reaction is written without any implication as to the nature of the receptors themselves. In this case, the degree of block would be related to the number of receptors occupied. The reaction might also be represented by the fundamentally distinct process:

$$\text{Curare} + \text{Receptors} \rightarrow \text{New Products} \qquad (2)$$

In this reaction, the drug is destroyed at the site of action. The available evidence is heavily in favor of reaction (1) and, in general, pharmacologists have assumed it to be correct. The evidence in favor of reaction (1) can be summarized as follows:

(A) It has long been known that the action of curare is not permanent. Moreover, Ing and Wright (78) demonstrated clearly that the paralyzed frog sartorius would return to its initial state after the muscle bath fluid was changed.

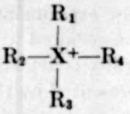
(B) The stability of onium ions predisposes to the truly reversible nature of their action. Alles and Knoefel (6) have pointed out that these compounds are not rapidly broken down in the body as is the ester linkage of acetylcholine. Their pharmacological actions are therefore more certain to be related to their physicochemical properties as ions, rather than to any hydrolytic or oxidative chemical reactivity.

(C) In a study of the effects of temperature changes on the action of curare (70), steady states were set up which could be approached from either side of the equilibrium. The degree of block at a given curare concentration was the same irrespective of whether the equilibrium was approached by the diffusion of curare into or out of the preparation.

(D) Finally, it was shown (70) that, in an isolated strip of rat diaphragm, the rate of diffusion of curare to and from its site of action determined the rate of paralysis and recovery of the preparation and that the rate of diffusion of curare into and out of the preparation was the same. This would seem to be proof of the truly reversible nature of the reaction.

The reversibility of the action of curare having been demonstrated, it is next of importance to answer two questions. How does such a union of drug and receptor block transmission, and what is the nature of the bond between curare and the tissues? Evidence concerning these two problems is complex, not very coherent, and interwoven with much tenuous hypothesis. The majority of relevant facts and hypotheses revolve around the possible role of the potassium ion in neuromuscular transmission, but no such role has as yet been proven for the $K^+$ ion.

The first definite suggestions for a mode of action of curare, supported by experimental evidence, were made by Ing and Wright (78) in 1932. They suggested that the bond between curare and its receptors was ionic in nature. They pointed out that the activity of fully saturated positively charged onium ions of the type



$$\begin{array}{c} R_1 \\ | \\ R_2 - X^+ - R_4 \\ | \\ R_3 \end{array}$$

was extraordinarily independent not only of the groups attached to the central atom (X) but also of the central atom itself. This atom could be either nitrogen, phosphorus or sulfur, the common factor in these compounds being their ionic nature. They also determined the temperature coefficient ($Q_{10}$) of the rate of paralysis of frog sartorius when it was soaked in a suitable solution of tetramethylammonium iodide. They estimated the $Q_{10}$ to be 1.5 and concluded that this value was consistent with the idea that the curariform ions were involved in an ion exchange reaction. That this $Q_{10}$ of 1.5 may be that of an uncorrected diffusion process (70) weakens the evidence for their hypothesis but does not invalidate it.

Ing and Wright (79) went further and suggested that, since onium ions were stable monovalent cations resembling the alkali metals, they might replace sodium or, more probably, potassium at the motor end-plate but would not be able to fulfill the function of the displaced ion.

Hypotheses similar to but not so definite as Ing and Wright's ion exchange reaction have subsequently been put forward by other workers. As a result of his studies on the liberation of potassium ions by muscle stimulated both directly and indirectly, Reginster (111) concluded that nerve stimulation liberated potassium ions which took part as transmitters in the series of reactions which occurred between the liberation of the acetylcholine and the contraction of the muscle fibers. The decisive part of the evidence which he submitted was the demonstration that the stimulation of the motor nerve to a muscle completely blocked by curare liberated a considerable amount of potassium, almost as much, in fact, as when the muscle was stimulated through its nerve in the absence of curare. He therefore postulated that the potassium was liberated from an organic potassium complex by the acetylcholine liberated previously by the nerve stimulation.

Cicardo and Moglia (31) agree with the conclusions of Reginster, in that they confirm the liberation of potassium by acetylcholine and by nerve stimulation. They were unable to confirm the liberation of potassium by nerve stimulation when the preparation was completely blocked by curare.

In a carefully controlled and executed series of experiments, Wood, Collins and Moe (128) have shown that the potassium that escapes when a muscle is caused to contract is not dependent on functional nerve endings. They demonstrated that potassium loss from muscle commences within a very short period after the start of the contraction and that this loss is about the same, whether the motor nerve endings of the directly stimulated muscles were degenerated or blocked by curare, or whether the muscle was stimulated normally through its nerve. The effect of curare in preventing potassium loss from a nerve-muscle preparation stimulated through its nerve has also been confirmed by Fenn, Koeneman and Sheridan (54).

A consideration of recent work (19, 35, 36, 47, 61) on the exchange of potassium between muscle and its environment leaves little doubt that the source of potassium which is lost when a nerve-muscle preparation is stimulated is the large potassium store inside the muscle fiber. Moreover, if potassium is a neuromuscular transmitter and if it is liberated at the end-plate during nerve stimulation, it would, as Fenn (53) has pointed out, be exceedingly difficult to detect this small amount against the background of the potassium known to be lost from the muscle. This criticism applies, as Fenn has also indicated, to the experiments of Szent-Györgyi, Bacq and Goffart on the use of veratrine to detect potassium as a transmitter in the perfusion fluid from stimulated frog's legs (117).

It would appear to the reviewer, therefore, that the liberation of potassium at the motor nerve endings, as a link in the chain of neuromuscular transmission, has not yet been detected and that, so far, there is no direct evidence that such an event occurs. Those theories of curariform action based on the liberation of potassium rest on an inadequately demonstrated foundation. Nevertheless, there is some evidence that the potassium ion has a role in normal neuromuscular conduction.

Interest in the role of potassium at synapses seems to have started with the discovery (73) that vagus stimulation liberated potassium ions from the heart. It has also been known for some time that KCl could cause frog rectus muscle to contract (28). Feldberg and Vartianen (52) showed that small amounts of KCl added to the fluid perfusing a superior cervical ganglion could provoke a discharge of impulses from the ganglion cells. This observation led Laurent and Walter (90) to administer KCl to myasthenics. They reported that it caused some improvement in the clinical picture in these patients. In 1936, Brown and Feldberg (22) demonstrated that potassium ions could cause ganglion cells to discharge even after the perfused ganglion had been paralyzed by curare with respect to both preganglionic stimulation and acetylcholine. About the same time, Wilson and Wright (126) demonstrated the anticurare action of KCl on cat gastrocnemius.

The actions of potassium on voluntary muscle and on autonomic ganglion cells resemble each other. Close arterial injection of potassium ions stimulates voluntary muscle even after the muscle is fully paralyzed by curare (21). Studies on single muscle fibers of the lizard (24) have shown that the end-plate region is the part of the fiber most sensitive to potassium ions. Kuffler (86) states that in frog muscle the end-plate region is no more sensitive to the stimulating action of the potassium ion than the rest of the fiber. The fact, already noted, that close arterial injection of KCl can cause voluntary muscle to contract, whereas the addition of enough KCl to inactivate completely a slip of rat diaphragm (69) *in vitro* does not result in any detectable contraction, suggests that the rate of change of potassium ion concentration at the site of action is important. This point does not seem to have been studied, and no adequate attempt has been made to control it in the studies on the stimulating action of potassium mentioned above.

More recently, the interaction between the potassium ion and curare has been studied directly and quantitatively on the isolated rat diaphragm. Early in the attempt to measure the temperature coefficient of the rate of paralysis due to curare, it was noted (68) that not only was the rate of paralysis slowed by a fall of temperature of about 10°C. but that the steady state or equilibrium potency of curare was reduced also. The investigation of this phenomenon led to the study of the antagonism between curare and the potassium ion (110). Equilibria were set up in which the potassium concentration required to antagonize a given dose of curare was measured as a function of the curare concentration. It was shown, by extrapolation, that at constant temperature, in the absence of potassium, curare had no naturally-occurring antagonist and that, therefore, the curare ions and the potassium ions seemed to be competing for the same receptors. These studies were repeated, confirmed and extended by Holmes, Jenden and Taylor (71) who showed, in addition, that the curare-potassium equilibria being studied were reversible and that the antagonistic action of a fall of temperature of about 10°C. on curare could be quantitatively accounted for by the fact that the naturally occurring potassium ions in the preparation were more potent antagonists to curare at the lower temperature.

Below about 26°C. (69, 70) any further fall of temperature causes an increase in potency of curare. Moreover, potassium ions in this latter temperature range augment the action of curare. It would appear, therefore, that the complex effect of a fall in temperature in causing first a reduction in the potency of curare, and then an increase, can be accounted for in terms of the action of the temperature reduction on the potassium-curare interaction at the motor end-plate. Detailed attempts to explain these effects are speculative at present and will not be dealt with here.

The discovery of the neuromuscular blocking action of a series of polymethylene bistrimethylammonium ions of the type $(CH_3)_3N^+—(CH_2)_n - {}^+N(CH_3)_3$, has opened up a new field of chemical and pharmacological investigations. The most striking feature of this group is the way they differ from curariform compounds; so great, in fact, are these differences that considerable doubt has been cast on the idea that they are curariform. Preliminary reports of their discovery were made simultaneously by Barlow and Ing (8) and by Paton and Zaimis (105). More detailed reports (9, 106) have also been published.

The pharmacological actions of these compounds depend greatly on the length of the polymethylene chain, and for a given compound sensitivity varies considerably from species to species. In general, depending on chain length, the compounds block ganglia, block neuromuscular transmission, and have some muscarinic, nicotinic-stimulating and anticholinesterase action. The ganglionic blocking action is maximal at $C_5$–$C_6$, and the neuromuscular blocking action shows an optimum at $C_{10}$.

If, when we use the term curariform, we mean that a given compound has the same mechanism of action on a molecular level as *d*-tubocurarine, then it will indeed be difficult to regard $C_{10}$ as belonging to this class.

There are points of similarity between $C_{10}$ and *d*-tubocurarine chloride (106). Neither blocks conduction in motor nerves, neither stops the response of the muscle to direct electrical stimulation, and both block the effect of close arterial injection of acetylcholine without preventing the liberation of the latter by motor nerve stimulation. These similarities suggest that perhaps $C_{10}$ might be classified as curariform, but the fact that it is not antagonized by the anticholinesterases (106) raises serious objection to this at once. From the work so far published, it is clear that the more the action of $C_{10}$ is analyzed, the more it is found to differ from *d*-tubocurarine. Paton and Zaimis express the view that $C_{10}$ and *d*-tubocurarine act at different points in the junctional region and bring evidence to support this contention. They point out that a muscle partially blocked with $C_{10}$ responds to stimulation with a well sustained contraction, while in a partially curarized preparation the response falls off rapidly. Moreover, the fact that the anticholinesterases do not antagonize $C_{10}$ suggests that $C_{10}$ and acetylcholine are not entering into the same type of competition as curare and acetylcholine. The problem is complicated by the fact that under suitable circumstances $C_{10}$ and *d*-tubocurarine can antagonize each other, and by the fact that $C_5$ can antagonize $C_{10}$ in concentrations which by themselves have no blocking action. Moreover, the potassium ion which has an important antagonistic action on curare does not antagonize $C_{10}$.

Recently, Burns and Paton (26) have stated that the fundamental action of $C_{10}$ is to depolarize the muscle fiber membrane at the end-plate. This depolarization spreads beyond the region of the end-plate and forms a ring-like area of inexcitable membrane about 2 mm. long around the fiber. The result of this is neuromuscular block.

A similar type of effect has already been ascribed to acetylcholine (23), and Paton and Zaimis (107) point out that $C_{10}$ can be regarded as having an action like acetylcholine after eserine. These authors further point out that *d*-tubocurarine acts by preventing acetylcholine from depolarizing the muscle membrane at the region of the end-plate and suggest that the term curariform should be restricted to those drugs which act simply by raising the threshold of the motor end-plate to acetylcholine.

This method of distinction between drugs which act like *d*-tubocurarine and those which act like $C_{10}$ will prove useful in the future, but it is doubtful if it will be absolute. From the models which Burns and Paton have set up for the actions of $C_{10}$ and *d*-tubocurarine, it follows that anticholinesterases should not antagonize decamethonium; in fact, they might be expected to intensify the block slightly. It is a simple matter to show (82) that the blocking action of carbaminoylcholine and nicotine on the rat diaphragm can be antagonized by eserine. The well known "nicotine" stimulating action of both carbamylcholine and nicotine suggest that they act like $C_{10}$, while their antagonism by eserine suggests that they are curariform. Perhaps these compounds have mixed actions, or it may be that the insensitivity of the rat diaphragm to $C_{10}$ is responsible for the phenomenon.

If the sole action of a pure curariform drug is to prevent acetylcholine from depolarizing the muscle membrane at the end-plate, then it should have no nicotinic stimulating action; rather, it should antagonize drugs with these actions. It is possible that many of the drugs heretofore regarded as curariform may resemble $C_{10}$ more closely in their mode of action.

It has been suggested that acetylcholine and $C_{10}$ act at the same point (107) and that *d*-tubocurarine prevents the action of these two substances. It seems that the receptors for *d*-tubocurarine, since they may be between the point of liberation of acetylcholine and its site of action, have a function in normal neuromuscular transmission which has not yet been defined. If we assume that *d*-tubocurarine blocks the diffusion of acetylcholine through some structure, we are faced with the difficulty of accounting for the forces which hold *d*-tubocurarine in place, because a space for diffusion could hardly form an ionic bond with a quaternary ion. On the other hand, there is no evidence at all for the existence of an acetylcholine carrier with which curariform drugs could combine.

It has been noted earlier that the $K^+$ ion does not antagonize $C_{10}$; in fact, large changes in the $K^+$ ion concentration have little or no detectable effect on a partial $C_{10}$ block of the rat diaphragm *in vitro* (82). This suggests that the site of action of the $K^+$ ion's antagonism to *d*-tubocurarine is central to the muscle membrane.

The interactions between drugs which act like $C_{10}$ and those which act like *d*-tubocurarine are complex (80, 107, 127) and have not yet been measured.

### *The Structure of Curariform Drugs*

One of the functions of wave mechanics is to explain the phenomena of chemistry on the deepest possible level. Developments in the field of theoretical physics in the last quarter century have made it possible to attempt to describe, explain and account for the structure of onium ions in terms of the distribution of atoms and bonding electrons within the molecule. No attempt will be made to set out a complete and accurately documented account of the developments in wave mechanics which have made such a description possible; rather, immediate reference will be made to the most recent and authoritative sources.

Pauling's monograph (108) contains a general account of the important work up to 1944. Since that time a series of monographs and reviews has appeared, the most important of which, in chronological order, would appear to be those of Palmer (102), Heitler (63), Bowen (17), Hume-Rothery (74), Mott and Sneddon (98), Rice and Teller (113) and Noller (101). The review of Coulson (37) is of special importance and interest in connection with the subject matter of this article.

### *The Structure of the Nitrogen Atom*

It is now possible, from a consideration of the electrons of the nitrogen atom available for bond formation, to account for many interesting structural features of tertiary amines and their ionic quaternary ammonium derivatives. The nitrogen atom in the ground state consists of a nucleus and seven extranuclear electrons distributed in the energy levels represented by Fig. 1. There are two electrons with opposite spins in the lowest energy level (1s electrons) two electrons with opposite spins in the next higher energy level (2s electrons), and three 2p electrons in an energy level a little above the level of the 2s electrons.

Each separate orbital can take a maximum of two electrons, and the spins of these must be opposite. The electron distribution can be represented as $(1s)^2$, $(2s)^2$, $(2p)^3$. The 1s and 2s orbitals are spherically symmetrical, and we need not examine them in detail here. Excellent pictorial representation can be found in the sources previously indicated (37, 74). From the standpoint of the structure of the tertiary amines and their quaternary derivatives, however, the spatial distribution of the three 2p electrons in the nitrogen atom is fundamental (37, 97).

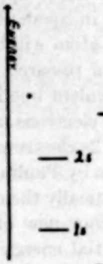


FIG. 1

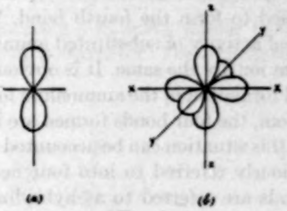


FIG. 2

FIG. 1. Energy levels of electrons in the nitrogen atom

FIG. 2. Atomic orbitals: (a) single 2p orbital; (b) the 3 possible 2p orbitals

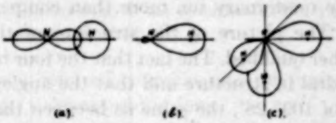


FIG. 3. Molecular orbitals: (a) the 1s orbital of the H atom and a 2p orbital of the N atom; (b) orbital of single N—H bond; (c) molecular orbitals of three N—H bonds in $NH_3$.

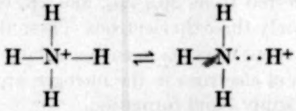
Each 2p orbital is roughly dumbbell in shape (Fig. 2a), the distribution and motion of the electron or electrons in each orbital being such that they are practically confined to such a space. Three 2p orbitals occur; they are arranged at right angles, and are referred to as $2p_x$, $2p_y$, and $2p_z$ orbitals (Fig. 2b). In the nitrogen atom there are only three 2p electrons. These three 2p electrons arrange themselves so that there is one electron in each available 2p orbital. The approximate spatial distribution of electrons in the nitrogen atom is now accounted for, and we may proceed to study bond formation.

Let us, first of all, consider the formation of the ammonia molecule by uniting one nitrogen atom to three hydrogen atoms. Each hydrogen atom has one 1s electron distributed around its nucleus in a spherically symmetrical orbital (Fig. 3a). The principle of maximal overlap states that the strongest bond (that with the lowest potential energy) will be formed when the electron orbitals of the atoms concerned have the maximal degree of overlap. It is obvious that this is achieved when the N-H bond is formed along one of the 2p orbital axes (Fig. 3b). The formation of three such bonds between the nitrogen atom and the hydrogen atoms gives the ammonia molecule (Fib. 3c), thus accounting for the pyramidal structure of the latter. That the ammonia molecule has indeed this structure is well known (97).

Experimentally the bond angles in ammonia have been shown to be 107° (129) rather than the 90° required by three mutually perpendicular 2p orbitals. The increase in angle is mainly due to repulsion between the three H atoms.

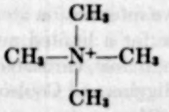
Let us next consider the formation of a quaternary ammonium ion by uniting the ammonia molecule with a hydrogen ion. The fact that all three 2p orbital electrons of the N atom are no longer available but are involved in covalent bonds with hydrogen atoms means that two 2s electrons from the N atom will have to be used to form the fourth bond. We know from the classical research on the optical activity of substituted ammonium ions that all four covalent bonds in an onium ion are the same. It is obvious, therefore, that while the electrons used for bond formation in the ammonium ion are the three 2p and two 2s electrons of the N atom, the four bonds formed are identical. It has been shown by Pauling (108) that this situation can be accounted for if we combine mathematically the orbitals previously referred to into four new identical orbitals. The four new identical orbitals are referred to as hybridized orbitals, and the potential energy of the system can be shown to be lower than it would have been if all the four bonds had not been the same. The energy required to raise the s electrons to form the hybridized bonds comes from the energy set free by bond formation, and the increased stability of the quaternary ion more than compensates for the energy lost in hybridization. The picture of the structure of the ammonium ion so described must be further qualified. The fact that the four bonds are equal means that the ion is tetrahedral in structure and that the angle between the bonds is the tetrahedral angle of 109° 28′, the same as between the bonds of the carbon atom. It might be added (97) that the widening of the bond angles in the ammonia molecule from 90° to 107° is associated with some of the hybridization of the 2s and 2p electrons referred to above.

The model of the quaternary ammonium ion, as we have so far described it, is inadequate, as Pauling has pointed out (108). In the language of resonance, each individual bond is a hybrid of the structures



$$\begin{array}{c}\text{H}\\ |\\ \text{H—N}^+\text{—H}\\ |\\ \text{H}\end{array} \rightleftharpoons \begin{array}{c}\text{H}\\ |\\ \text{H—N}\cdots\text{H}^+\\ |\\ \text{H}\end{array}$$

This ionic covalent bond resonance can be alternatively described by saying that each bond has a given percentage ionic character, and Pauling estimates this at 20 per cent for each N-H bond in NH$_4^+$. It follows, therefore, that the positive charge, tacitly localized by classical theory to the central atom, is really spread out over the whole ion, and it appears that about one fifth of it is localized to the central N atom. These considerations clearly apply to all saturated quaternary ammonium ions. In ions such as



$$CH_3-\overset{\overset{CH_3}{|}}{\underset{\underset{CH_3}{|}}{N^+}}-CH_3$$

the methyl groups are attached to the central N atom by four separate, identical, tetrahedrally directed, two-electron bonds. In any onium ion of the type $R_4N^+$ the extent of the central charge spread will depend on the dipole moment of each N-R bond which, in turn, will depend on R.

The description given above must be qualified in several respects when two of the four bonds in an onium ion together form a double bond, as in a pyridinium or quinolinium ion. Here one 2p orbital of the N atom is involved in forming half of the double bond, and the other three bonds are s-p hybrids, similar to those in the saturated ion except that they have more s character. The hybridization in the unsaturated ions is referred to as trigonal, as opposed to the tetrahedral s-p hybridization found in the saturated ions.

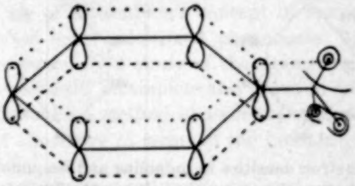


FIG. 4. Molecular orbitals and hyperconjugation in the methylpyridinium ion. The dotted lines indicate the lateral overlap of the 2p orbitals. The method of representation is similar to that used by Evans and de Heer (51).

In benzenoid systems such as the methylpyridinium ion (Fig. 4) the classical double bond does not occur. The benzenoid character of this structure is due to the overlap of each 2p orbital with its two nearest neighbors.

The structural difference between the saturated ions and those with a 2p orbital in a double bond or benzenoid ring has at least two consequences. Firstly, the greater mobility of the mobile π-electrons in the double bond causes it to be polarized towards the positively charged N atom and, hence, to have a charge-spreading effect; this will be especially marked when the 2p orbital of the N atom is part of a benzenoid system. The positive charge in this case will be more delocalized than in a saturated molecule. Secondly, the presence of a non-hybridized 2p orbital on the N atom predisposes it to hyperconjugation with any attached methyl group. Hyperconjugation (48, 99) in this case is due to the interaction of the electrons of the ring and the bond electrons of the methyl group (Fig. 4); it results in a further charge delocalization and hence a reduction of the charge on the N atom.

Qualitative correlations between electron distributions of the type described above and molecular properties, such as dissociation constants, dipole moments, ease and position of substitution of reagents, have frequently been made in the past. More recently, quantitative information about bond orders and charge distributions has become available for a limited number of molecules. Such data have been computed for pyrrole, indole, carbazole, pyridine, quinoline, isoquinoline, and acridine by Longuet-Higgins and Coulson (91). Figures for aniline have also been provided by Dewar (49).

It is fortunate that this series includes quinoline and isoquinoline, for quaternary condensation products of these and their tetrahydro derivatives have been assayed for curariform activity by Ing and Wright (78), and Collier and E. P. Taylor (33, 34) have studied some of their polymethylene-bis-quaternary compounds. Moreover, the tetrahydroisoquinoline ring system is found in *d*-tubocurarine.

The mobile of $\pi$-electron densities at the atoms of quinoline and isoquinoline are shown in Fig. 5 (91).

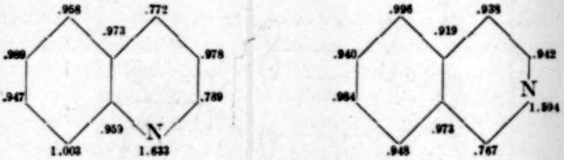


FIG. 5. Mobile or $\pi$-electron densities in quinoline and isoquinoline. The net charge at each atom can be obtained by subtracting the value in the figure from 1. For example, the charge at position 4 in quinoline is +0.23e while that at position 1 in the same molecule is −0.633e.

In benzene each carbon atom contributes one p electron to the total mobile sextet. The $\pi$ electron density at each carbon atom of 1.0 means that the net charge here is zero. Mobile or $\pi$ electron densities of less than one (X for example) mean a positive charge of magnitude (1—X) times the electronic charge, whereas electron densities of more than 1 (Y for example) mean negative charges of (Y—1) times the electronic charge. It is clear at once that the charge differences between different atoms in quinoline and isoquinoline are large and very interesting.

In both quinoline and isoquinoline the charges located on the N atoms are the largest in each molecule and are negative. The charge on the N atom in quinoline is greater than that on isoquinoline, a point reflected by the fact that quinoline is the stronger base of the two (121). In the remainder of these atoms the electron densities are all positive. They lie close to unity, except at positions 2 and 4 in quinoline and position 1 in isoquinoline, where larger positive charges are located. Finally, as we move from atom to atom in these molecules along any consecutive arrangement of bonds, the charges alternate in intensity (41).

### *Structure-Action Relationships*

The scarcity of successful attempts to relate chemical structure to pharmacological action is adequate evidence of the difficulty of the subject. Some of the difficulty can be defined and the rest better appreciated if we attempt to analyze the general problem.

We cannot relate the structure of a molecule directly to the gross pharmacological action it produces in an organism. The action of a drug will be a function of its chemical and physical properties which, in turn, may be correlated with its structure. If we accept the principle of analyzing the problem into a sequence of immediate causes, we still cannot relate action to properties because the action we measure should be correlated with the biochemical reactions which are involved. These latter can, in turn, be related to molecular properties. We can represent this analysis as follows:

Crude Structure → Molecular Properties
→ Fundamental Biochemical Reaction→ Gross Pharmacological Action

### *Relation Between Structure and Molecular Properties in Compounds*

It is clear that the representation of organic molecules by letters of the alphabet joined by short straight lines was never meant to be either complete or accurate. Pauling's use of Heisenberg's concept of resonance extended the use of classical notation to cover additional phenomena, but the concept of the resonance hybrid generated by a study of the resonating forms depicted in the classical manner was difficult, incomplete and prone to error. At present, molecular oribtal theory provides a method of understanding and representing many of the phenomena of chemistry in terms of the fundamental concepts of probability theory.

Even with improved methods of molecular representation, the relation between structure and properties in organic compounds is a formidable problem in physical chemistry. Its solution is an obligatory step in any complete structure-action relationship. The problem is complicated by the difficulty of deciding which molecular properties are pharmacologically important, and quantitative assessment of the importance of any one property is complicated by the presence of the others.

### *Relation Between Molecular Properties of a Drug and the Reaction in Which It Participates*

Generally speaking, little is known of reaction mechanisms of drugs on the molecular level. Nevertheless, a consideration of the problems involved is useful from the experimental standpoint. Drug-receptor reactions can be divided into two groups. In the case of reversible drug-receptor reactions, it would seem that the dissociation constant of the reaction

$$\text{Drug} + \text{Receptor} \rightleftharpoons \text{Drug-Receptor Complex}$$

would be the most fundamental measure of activity. In the case where the drug-receptor reaction is such that the drug is destroyed,

$$\text{Drug} + \text{Tissue Constituents} \rightarrow \text{New Products}$$

it would seem that the velocity constant of the reaction would be the most useful measure of activity. The fundamental difference between these two basic types of reaction can be appreciated better if we consider the effect of changes of temperature on them.

If the drug-receptor reaction is a reversible dissociation, we would expect its equilibrium constant to be related to its heat of reaction by the van't Hoff isochore

$$\ln K = -\frac{\Delta H}{RT} + C$$

where K is the dissociation constant, $\Delta H$ is the heat of the reaction and C is a constant. Since the heat of the reaction can be regarded as a constant, ln K plotted against $\frac{1}{T}$ should be a straight line of slope $-\ \Delta H/R$.

In the case where the important quantity is a velocity constant, its temperature dependence can be expressed by the Arrhenius equation,

$$V = Ae^{-E/RT}$$

where A is a constant and E is the activation energy. In this case, a graph of ln V against $\frac{1}{T}$ should give us a straight line of slope $-\ E/R$. In both types of mechanism, a study of the effect of temperature on the action of the drug should provide fundamental information. Unfortunately, the nature of biological reactions imposes serious limitations on the study of the effect of temperature on them. The maximum possible temperature range available for studies of this type is less than 40°C. In practice, the action being studied may be confined to a much narrower temperature range. The practical importance of the distinction between the two types of reaction mechanism can be understood if we consider the consequences of using an assay procedure which tacitly assumes a reaction mechanism other than that which occurs. Before we study this, it will be useful to comment on the relationship between the basic drug-receptor reaction and its pharmacological result.

### *Relationship Between Basic Drug-Receptor Reaction and Macroscopic Pharmacological Result*

Even in the simplest case, it is necessary to have quantitative knowledge of the relationship between pharmacological action and the dissociation constant, velocity constant or partition coefficient of the basic reaction producing it before we can make deductions about these reactions from their observed biological results. The relation between basic biochemical action and the observed macroscopic pharmacological result could be highly complex and is probably never simple. Nevertheless, knowledge of this connection is necessary before we can complete the relationship between the structure of a compound and its action.

If a drug acts by combining reversibly with its receptors, it should be possible to set up a preparation in which a sate of true equilibrium between drug and receptors exists. In this type of experiment, variation in pharmacological action with alteration in drug concentration should supply information on the contypes of reaction can be appreciated better if we consider the effect of changes of temperature on them.

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$$\text{Drug} + \text{Receptors} \rightarrow \text{Drug-Receptor Complex}$$

to equilibrium. The result of such an assay will depends on the rate-limiting factors in the reaction. If this rate-limiting factor be diffusion, then the result of the assay should contain a term proportional to the ratio of the diffusion rates of the two compounds. The result will not be wholly dependent on the pharmacological potency of the compounds and, in any case, its numerical value will depend on the dose of the standard used and hence will be without significance.

If we attempt to assay two drugs against each other which act according to the equation

$$\text{Drug} + \text{Receptors} \rightarrow \text{New Products}$$

by setting up a steady state, we will not be measuring the velocity constant of the reaction. This is because such steady states depend for their existence on subsidiary reactions which maintain the drug concentration at a steady level. It follows that the numerical result of such an assay would be largely dependent on such subsidiary reactions and hence not a true measure of drug activity.

It is clear that any absolute method of comparison of the pharmacological activities of two drugs must take their mode of action into account in order to ensure that the basic properties of the drugs responsible for their pharmacological action are being assayed.

### *Structure-Action Relationship in Monoquaternary Ammonium Ions*

Structure-action relationships in the monoquaternaries is a wide field, and discussion here will be confined entirely to curariform action. Other wider aspects of the problems have been considered by Alles (3–5). Owing to the several possible pharmacologically active properties which an onium ion might have (6, 78), it should clarify our views if we mention those that have been shown to be unimportant.

The fact that curariform activity is in many cases remarkably independent of the various groups attached to the central nitrogen has been known since the time of Boehm. That it is also independent of the central atom is proven by the demonstration that phosphonium, sulphonium and arsonium ions can also block neuromuscular transmission.

Ing and Wright (78) showed that curairform activity in simple onium ions was not related to adsorbability on charcoal, to their water solubility, to their hydration or to their surface activity. Further, they pointed out that, except for the possibility that weakness of activity might be associated with a restricted range of cationic size, there was no relation between size and activity. It appears that this can be ruled out also, since many large inactive quaternaries are now known. Further, as these quaternary ions are completely ionized in solution, there can be no relationship between their activities and their dissociation, nection between basic biochemical action and pharmacological result. Let us consider the comparison of the activities of two drugs which act reversibly by measuring the rate of approach of the reaction

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The fact that curariform activity is in many cases remarkably independent of the various groups attached to the central nitrogen has been known since the time of Boehm. That it is also independent of the central atom is proven by the demonstration that phosphonium, sulphonium and arsonium ions can also block neuromuscular transmission.

Ing and Wright (78) showed that curairform activity in simple onium ions was not related to adsorbability on charcoal, to their water solubility, to their hydration or to their surface activity. Further, they pointed out that, except for the possibility that weakness of activity might be associated with a restricted range of cationic size, there was no relation between size and activity. It appears that this can be ruled out also, since many large inactive quaternaries are now known. Further, as these quaternary ions are completely ionized in solution, there can be no relationship between their activities and their dissociation, and, as mentioned earlier, their stability and true reversibility of action rule out hydrolytic or oxidative chemical reactivity.

As a result of these and other considerations, Ing and Wright (78), Ing (77) and Alles and Knoefel (6) concluded that activity is associated with some physicochemical property due to the ionic nature of these compounds.

### *The Charge Density Theory of Curariform Activity and the Relation between the Ionic Properties of the Quaternary N Atoms and Activity*

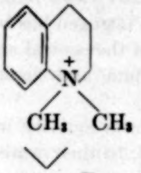
The above observations led Holmes, Jenden and Taylor (67) to consider the activities of the quaternaries studied by Ing and Wright in the light of their known wave mechanical structure. In the section on structure, Pauling's work on the charge distribution in $^{+}NH_4$ was briefly described, and it was pointed out that in $R_4N^+$ the charge distribution should depend on the relative electronegativity of R. The relative order of positive charge densities on the N atoms in tetramethyl-, tetraethyl-, tetrapropyl- and tetrabutylammonium ions was estimated from the relative polarity of the substituent groups which, in turn, were obtained from dissociation constants of the corresponding carboxylic acids and mono-, di- and trisubstituted amines. Activity was seen to be related to the amount of charge on the central N atom, the charge being most distributed and activity lowest in $(C_2H_5)_4N^+$.

The ethyl group anomaly noted in the activity of tetraethylammonium ions, in the dissociation constant of propionic acid, and the mono-, di- and triethylamines has also been detected in certain organic reactions (1, 2, 7, 20, 50, 62). No adequate explanation of the anomalous polarity of the ethyl group is yet available.

Arguing from the known dipole moments of bonds between hydrogen on the one hand and nitrogen, sulfur, phosphorus and arsenic on the other, it was also pointed out (67) that activity is parallel to central positive charge in oniom ions containing these atoms. Bond dipole moments are more reliable estimates of charge distribution than the crude relative inductive effects used in the previous argument.

Ing and Wright (79) showed that the order of activity in some quinolinium and tetrahydroquinolinium ions was as follows:

I



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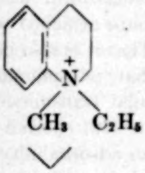
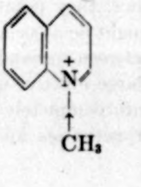
II



III



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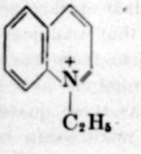
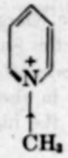
IV



Compounds III and IV constitute an important exception to the idea that ethyl derivatives are less active than the corresponding methyl compounds. In the pyridinium compounds V and VI, the ethyl compound is also more active than the methyl (79), while in morphine, strychnine and nicotine, the metho salts are more active than the etho salts. Ing and Wright have generalized this

V



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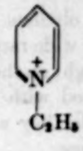
VI



latter complexity by suggesting "that when nitrogen forms part of a ring system replacement of methyl by ethyl in the quaternary salts leads to an increase in curariform properties if the ring be aromatic and to a decrease if the ring be saturated."

We know, from the theoretical and experimental work referred to in the section on structure, that methyl groups hyperconjugate with aromatic but not with saturated rings and that ethyl groups do not do so to any appreciable extent with either. This hyperconjugation is accompanied by an electron drift towards the ring and is indicated by the arrow in III and V above. The difference in activity between III and IV and between V and VI is again associated with reduction in positive charge on the onium nitrogen.

In Figure 5 we see that the nitrogen atom in quinoline is the site of a negative charge of magnitude 0.633e. This is chiefly due to the electronegativity of the N atom itself and to the fact that it is situated in a highly polarizable unsaturated ring. Moreover, it is clear that the positive charge developed by the movement of electrons towards the N atom is spread out over the whole ring system, being greatest at position 4 and least at position 9. When this N atom is quaternized, as in methyl-(III) and ethylquinolinium (IV) the positive charge will be reduced by at least half of its original amount. No such very large reduction can occur in the 1, 2, 3, 4-tetrahydro derivative, for the $\pi$ electrons form no bonds with the N atom in this case.

Finally, in this connection Von Oettingen and Eveleth (119) have shown that the curariform actions of choline (VII) are less than that of the monochloroethyl trimethyl ammonium ion (VIII). The electron draw of the chlorine atom increases the positive

$(CH_3)_3N^+{-}CH_2{-}CH_2{-}OH$ VII. $\qquad$ $(CH_3)_3N^+{-}CH_2{-}CH_2{-}Cl$ VIII.

charge on the N⁺ atom Conversely, in betaine, $(CH_3)_3N^+\cdot CH_2COO^-$, we would expect the negatively charged carboxyl group to act as a powerful electron repellent and so reduce the positive charge on the N atom. Pharmacologically, this compound is remarkably inactive; Hunt and Renshaw (75) and Renshaw (112) found curariform action to be absent. The electron repulsion in the ionized carboxyl group of betaine can easily be converted by esterification into an electron attraction. That the ester of betaine is more active than betaine itself has been shown by Hunt and Renshaw (75). The abolition of carboxyl group negative charge by amide formation has also been shown to restore some activity (76).

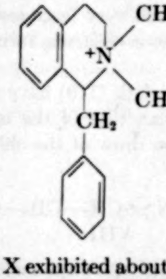
### *Other Factors Contributing to the Activity of the Monoquaternaries*

It is, however, quite obvious that simple charge density considerations do not suffice to explain the wide range of activities found in the monoquaternaries. Nor do they throw any light on the differences in activity of the α- and β-canadine methochlorides, associated with differences in their optical activities, studied by Laidlaw (87). Nor do they account for the high activity found in certain complex monoquaternaries.

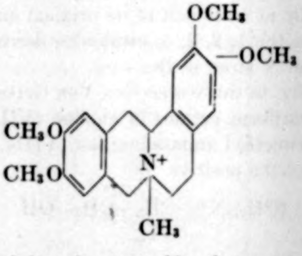
The union between a quaternary nitrogen atom, which is part of a large and complex molecule, and a receptor could not be independent of the interaction of the rest of its molecule and the atomic lattice surrounding the receptor. Van der Waals attraction forces would occur and would depend on the dipoles present in, and on the polarizability of, both structures. There is no reason why α- and β-canadine methochlorides should have equal actions, since one might interact more effectively with the receptor surface.

Moreover, since Van der Waals forces are effective only over very short ranges, a high degree of specificity is to be expected if the structure of the molecule, in general, is to result in maximal activity. Nothing is known of the requirements of this problem, and it would seem that further investigation into it will have to be empirical. Extensive lists of compounds and their activities have been collected by Craig (43). Since this review, several papers have appeared in which various type structures have been tested for curariform activity. Craig and Tarbell (44) have studied quaternary ions of the benzyl tetrahydro-isoquinoline type because this ring system occurs in *d*-tubocurarine. The most active quaternary in this series was IX which had an activity of 1/75 of *d*-tubocurarine when tested on mice.

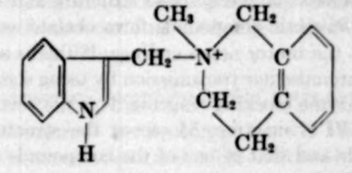
IX



X



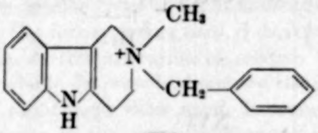
Compound X exhibited about the same activity when tested by the same method. In the third paper in this series Craig and Tarbell (45) have studied the 3-indolylmethylamine series because of possible relation to C-toxiferine I. Compound XI was the most active here, being effective in a dose of less than 16 mg./kg. by intraperitoneal injection in mice. They point out that the 3-indolylmethyl group greatly enhances curariform activity, because N,N-dimethyl-1,2,3,4-tetrahydroisoquinolinium iodide was ineffective in over 10 times the effective dose of XI.



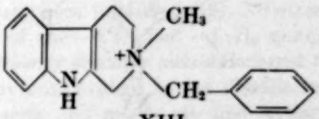
XI

This compound XI raises the interesting question as to whether the indole nitrogen atom has curariform activity of its own or whether the 3-indolylmethyl group acts in some other way. If it has curariform activity, it will be very unusual because secondary amines with this action appear to be almost unknown.

Papers four and five in this series (12, 46) consider compounds in the piperidine series and in the quinolizidine series, and papers six (10) and seven (11) deal with some β- and γ-carbolines and 1-skatolisoquinoline derivatives. Compounds XII and XIII were the most active of the β- and γ-carboline series, both being about one fifth as active as *d*-tubocurarine in the frog. Incidental to this

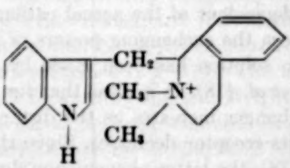


XII



XIII

work, it was noted (10) that XIV was of about the same activity as XII and XIII on the frog.



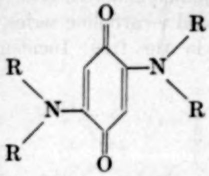
XIV

Cavallito, Soria and Hoppe (27) have prepared two series of quaternary compounds, the first related to XV and the second to the corresponding mono-N compound.

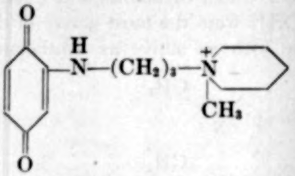
Relative to compounds in the same class, high activity was noted in the diquaternaries, monoquaternaries and in the unquaternized mono- and bis-alkylaminobenzoquinones. The monoquaternary ion XVI caused head-drop in the rabbit in a dose of 0.074 mg./kg. This is a very high activity for a monoquaternary. They also point out that in ions of this type, activity does not vary much with changes in chain length between the nitrogen atoms.

Cavallito *et al.* suggested that the high activity of these agents can be associated with involvement of the quinone structure and that it is possible that these quinone curarimimetic compounds form chelate complexes with metalloporphyrin groups at the motor nerve endings. If this is so, it might be possible to interfere with neuromuscular transmission by using some of the highly specific metalloprophyrin enzyme blocking reagents. It is not certain that the secondary nitrogen atom in XVI is inactive. Moreover the structure of *d*-0,0-dimethyltubocurarine chloride and that of one of the compounds studied by Collier and Taylor (34) (Table I) show that the quinone structure is not necessary for high activity.

The agents studied by Cavallito *et al.* were assayed by the inclined screen mouse method and the rabbit head drop method. Unfortunately, neither of these methods is specific for curariform activity, and the possibility remains that in some of these compounds other pharmacological actions may have complicated the results. Experience suggests that this source of error is more likely to result in the case of amines than in the case of highly active quaternaries.



XV



XVI

### *Possible Significance of the Charge Density Theory*

According to Walton (124), the order of adsorption of inorganic ions on cation exchangers is independent of the actual cation exchanger used. Quantitative correlation between the exchanging powers of these ions and the radius of the hydrated ions in solution has been made by Jenny (81), Nachod and Wood (100) and Boyd *et al.* (18). It is clear that for cations of a given charge the affinity for the exchanger increases as the distance of closest approach of the hydrated ion and its receptor decreases. Since the quaternary ammonium ions are not hydrated (78), the latter phenomenon does not enter into our estimate of the size of these ions. Moreover, the size of the molecule containing the quaternary cannot be related to the ionic size of the positively charged nitrogen atom, since the latter is localized to a small part of the whole molecule. If the affinity of a quaternary ammonium ion for its receptor is, in part, related to the distance of closest approach which it can make to a receptor, then it does not seem unreasonable that quaternaries in which the positive charge is least spread out should have the highest affinities for their receptors. In fact, instead of referring to the closest approach of the ions, we might refer to the closest quaternary. They also point out that in ions of this type, activity does not vary much with changes in chain length between the nitrogen atoms.

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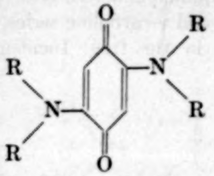
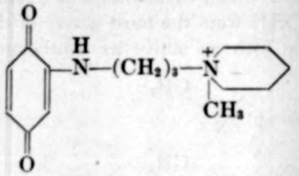


XV



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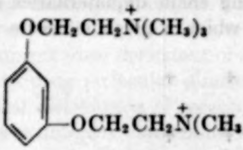
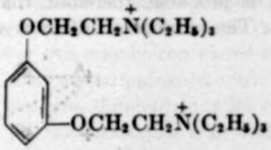
### *Limitations and Apparent Exceptions to the Charge Density Theory*

There appear to be exceptions to the charge density theory, all associated with differences between quaternaries substituted with methyl and ethyl groups. While it appears to be the case that, in ions of the type $-N^+R_3$, activity is greater when R is $-CH_3$ than when it is $-C_2H_5$, this is not always so. The compound XVII is less active than XVIII (15).


XVII


XVIII

Other similar cases of ethyl substituted onium compounds being more active than the corresponding methyl derivatives have been published (16). No explanation of this alteration of relative effects of the $-CH_3$ and $-C_2H_5$ groups is available. In organic chemistry the ethyl polarity anomaly already referred to does not always show itself. It seems to be most evident in the dissociation constants of the corresponding carboxylic acids and mono-, di- and tertiary amines. Difficulties of this type are likely to continue until we have a better understanding of the fundamental differences between ethyl and methyl groups.

A further, and perhaps more interesting exception, is furnished by the work of Lasslo and Kimura (89) who claimed that N-methyl-ementine-N,N-diethoethyl-sulfate was more active than N-methyl-emetine-N,N-dimethiodide; here the quaternary group is in a saturated ring (114). This also seems to constitute an exception to the rule put forward by Ing and Wright (*loc. cit.*).

Chase, Lehman and Rickards (30) also claimed that the etho quaternary salts of quinine were more active than the metho, but a reinvestigation by Marsh and Pelletier (96) suggests that the metho salts are the more active.

### *The Diquaternaries*

Considerable success has resulted from the inclusion of two quaternary groups in the same molecule and the empirical adjustment of the distance between them to the optimal value. From the theoretical standpoint, however, the problem is not simple. In spite of the fact that pharmacological activity is very sensitive to alteration of interquaternary group distance, we still have little idea as to what the average inter-receptor distance might be and no information approach of the charges, in which case it is obvious that localization is important, for the more the charge is localized the easier it will be to get it close to its receptor. The analogy between the *"in-vitro"* synthetic ion exchange and the biological processes occurring at the motor end-plate cannot be close because the crude granules of the former could not be compared with specific and highly organized structures found in living tissues.

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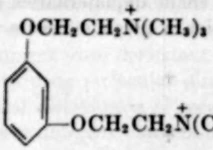
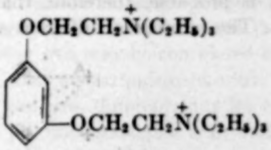


XVII



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If we consider two quaternaries separated by a long saturated hydrocarbon chain, it would appear that such a molecule could assume a very large number of configurations, coiled and otherwise. The interterminal group repulsion would, of course, tend to straighten the molecule (106). In the case of the simple dicarboxylic acids in solution—oxalic, malonic, succinic up to sebasic—the estimation of the intercarboxyl distance in solution by Gane and Ingold (57) did not show any marked tendency for the saturated hydrocarbon chains to form coils. It is probable, therefore, that the long chain diquaternaries are fairly straight. The number of different ways in which a given molecule with two active groups at a known distance apart can be fitted to an array of receptors is a problem in statistical mechanics and is too complex to be discussed here. It has been considered in general by Guggenheim (60) who refers to the earlier literature. A further point of interest is that the combination of a compound with an array of receptors may cover some of the latter or otherwise inactivate them without actually combining with them (65, 66)

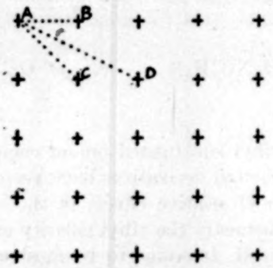


FIG. 6. Some possible arrangements of diquaternaries on a hypothetical square lattice.

We can perhaps obtain some idea of the problem by assuming an array of n receptors in a regular square lattice formation (Fig. 6).

If the distance between the active groups of the drug is equal to either the side A-B or the diagonal A-C of the smallest square, then the minimum number of drug molecules required for a complete occupation of the receptors will be n/2. Maximum drug adsorption associated with incomplete receptor occupation is, of course, possible when an unoccupied receptor is so surrounded with occupied receptors or covered that it cannot be bridge-linked with another receptor. Other positions of fit such as A-D are, of course, possible, and with larger drug intergroup distances the number of different possible positions increases. It is clear that for such a square array, drug activity should have optimum values when the interactive group distance corresponds to some direct or diagonal inter-receptor distance or multiple thereof. Moreover, if the inter-receptor distances were different in different muscles and if the receptor array were square, then it is conceivable that a drug which fitted best in position A-C in one muscle might fit best in position A-D in another. Under these circumstances, we would not expect the orders of activities of a series of double quaternary drugs to remain the same when assayed on different muscles. In fact, a drug which fitted well in one lattice might fit only with difficulty into another square lattice. Great divergencies of activity could, therefore, be expected from such a model.

It is exceedingly unlikely that the receptors for curare in a motor end-plate will be arrayed with the maximum possible degree of order, that is, in a square array. Let us, therefore, consider a completely random array. Constancy of molecular dimensions and of bond angles will set a lower possible limit for inter-receptor distance; no upper limit need be considered for the purpose of this argument since the extent of the receptor area may be considered large with respect to drug molecular dimensions. If such an arrangement were true and the spatial distribution of receptors truly random, different muscles could still easily have different numbers of receptors per unit area. If a truly random distribution, such as the above, held, but if the number of receptors per unit area varied from muscle to muscle, we would expect the paralyzing dose of any drug to depend on the muscle, and there would seem to be no reason why the order of activity of a group of diquaternaries should be the same in different muscles with different random distribution.

Considerations of this type of problem and of previous hypotheses of inter-group distance (109) led Kimura *et al.* (83, 84) to study the diquaternary compound synthesized by joining two atropine molecules together in various ways. They studied the atropine-like and curariform activity of the dimethiodide of the amyl diether of atropine, where two atropine molecules are joined through the tropic acid hydroxyl groups, and the pentamethylene and decamethylene bis atropinium di-iodides prepared by connecting two atropine molecules through their tertiary nitrogen atoms. Twinning of the atropine molecules increased both the atropine-like and the curariform activity. As determined by the head-drop method in rabbits, the decamethylene-bis-atropinium di-iodide was about five times more potent than the corresponding pentamethylene derivatives and about twice as potent as *d*-tubocurarine. The curariform activity of these compounds was antagonized by neostigmine. It would be important to know how much of the activity of such compounds is due to the diquaternary part of the molecule and how much is due to the tropic acid groups.

One of the most recent and interesting developments in this field is the synthesis of decamethylene $\alpha,\omega$-bis-6,7,8-trimethoxy-1,2,3,4-tetrahydroisoquinoliniummethiodide by Collier and E. P. Taylor (34). It abolished righting reflex in the rabbit in a dose of 20 $\mu$gm./kg. These authors express the view that in the rabbit this compound is the most active synthetic compound yet reported. It is antagonized by neostigmine and is about seven times as active as decamethonium iodide. Its synthesis resulted from a study of the effects of the introduction of methoxy groups into some quinoline and isoquinoline derivatives. These compounds (33, 34), some of which are shown in Table I, are probably truly curariform. Several were shown to be antagonized by neostigmine. Collier and Taylor confirm Ing and Wright's demonstration that 1,2,3,4,-tetrahydroquinolines are more active than the fully unsaturated parent compounds. They showed that the isoquinolines were, in general, more active than the quinolines and that substitution with methoxy groups in positions 6, 7 and 8 increased activity. The most obvious explanation of the effect of the addition of the —$OCH_3$ group in increasing the potency of both *d*-tubocurarine itself and of the compounds mentioned above is that these groups can become loosely attached to the receptor surface and, thereby, increase the drug:tissue affinity.

This assumption is difficult to accept. Table I shows that the 6,7,8-methoxy, 1,2,3,4-tetrahydro isoquinilinium ion is more active than the 6,7-methoxy compound which, in turn, is more active than the 6-methoxy compound. If the methoxy group increases drug activity by attaching itself loosely to the receptor surface, it follows that there are three sites close together on the receptor surface with the characteristic properties which enable them to bind methoxy groups. Moreover, similar effects occur in the quinolinium compounds, in spite of the fact that the position of the quaternary group relative to the methoxy substituted atoms has changed. To explain this, the existence of a small area of receptor surface which could unite with methoxy groups would seem to be necessary.

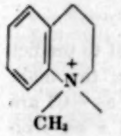
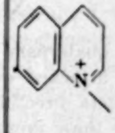
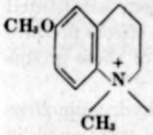
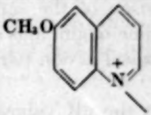
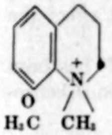
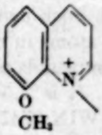
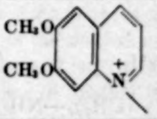
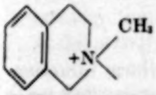
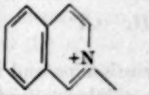
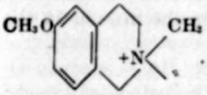
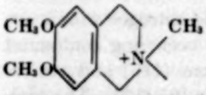
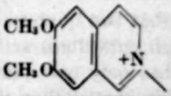
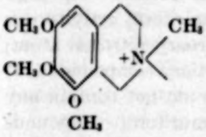
It is, of course, possible that the methoxy group acts by modifying the properties of the molecules to which it is attached. This involves us at once in the properties of this group and the effects of its substitution into a benzene ring. There is no quantitative theory of benzene substitution yet available; in fact, the deficiencies of our knowledge of the effects of substitution on the electrons are considerable. Some of these deficiencies have been defined (51). Interest in and the importance of this problem are such, however, that a very brief summary of the relevant literature may be useful in that it indicates how the problem might be investigated.

Important progress in the theoretical treatment of cyclic unsaturated hydrocarbons has been reported (39–41) and some of the gaps in our knowledge in this field have been defined (51). Moreover, the interaction of the four bonds at a carbon atom has been shown to be more extensive and flexible than was heretofore supposed (38).

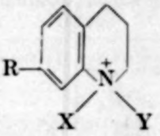
While it is impossible to predict quantitatively the effect of —$OCH_3$ groups on quaternary nitrogen atoms in quinoline, isoquinoline, and their tetrahydro derivatives, a theoretical consideration of the question suggests two possible lines of experimental analysis. The methoxy group will have two somewhat interdependent effects on the benzene ring to which it is attached. Its dipole moment (1.23 D aromatic) (59) is such that it will exert an electron-attracting inductive effect on the electrons of the ring, while one of the p orbitals of the oxygen atom will extend the conjugation path of the $\pi$ electrons of the ring.

## TABLE 1

*Curariform activities of a series of methoxy substituted quinolines and isoquinolines* (33, 34).

| GENERAL FORMULA = $Q—(CH_2)_{10}—Q$ WHERE Q IS HALIDE OF: | APPROXIMATE M.E.D. RABBIT | Q HALIDE WHERE GENERAL FORMULA IS $Q—(CH_2)_{10}—Q$ | APPROXIMATE M.E.D. RABBIT |
|---|---|---|---|
| | *Mg./Kg.* | | *Mg./Kg.* |
|  | 0.75 |  | 4.5 |
|  | 0.2 |  | >4.0 |
|  | 0.1 |  | 0.2 |
| | |  | 0.15 |
|  | 0.75 |  | >4.0 |
|  | 0.2 | | |
|  | 0.05 |  | >1.0 |
|  | 0.02 | *d*-0.0-dimethyltubocurarine chloride<br>*d*-tubocurarine | 0.01<br>0.1 |

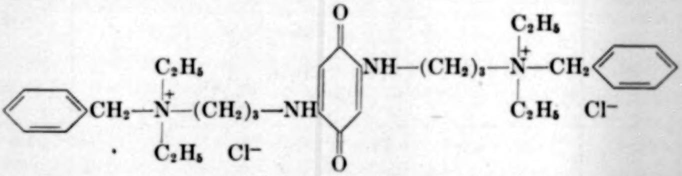
The resultant effect of a methoxy group at position R in



on the $\sigma$ bond leading to the quaternary nitrogen atom is very difficult to predict theoretically but could be attacked experimentally. For example, the main effect of the $NO_2$ group is meta directing, while halogen atoms such as —Cl are ortho para directing. Since these groups have opposite effects on $\pi$ electron distribution, a comparison of the activities of nitro and halogen substituted quinoline and isoquinoline onium ions should show whether such effects produce any alteration in biological action parallel to the known effect of these groups on such molecules.

It seems a pity that the pK values of the substituted tetrahydroquinolines and tetrahydroisoquinolines used to prepare the compounds mentioned in Table I were not measured. Such determinations would show what effect the substitutions had on the N atoms in these compounds.

One of the most active compounds prepared by Cavallito *et al.* (27) has been studied in more detail (72). It is 2,5-bis-(3-diethylaminopropylamino) benzoquinone-bis-benzylchloride (WIN 2747).



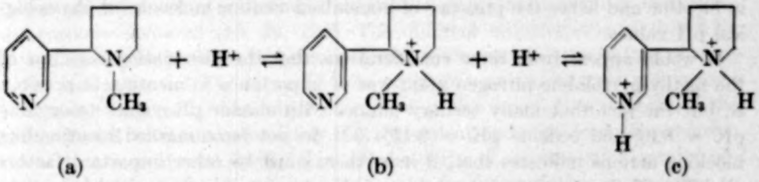
It is antagonized by neostigmine and appears to be truly curariform in action. Its duration of action is similar to that of *d*-tubocurarine. It is about five times as active as *d*-tubocurarine in the rabbit but less active in the mouse. In the dog it is about twice as active as *d*-tubocurarine.

### *The Nicotine Problem*

There is evidence that two quaternary positively charged nitrogen atoms are not necessary for high curariform activity. The naturally occurring compound calabash-curarine-1 has only one quaternary nitrogen atom (116) and yet its activity is considerably greater than that of *d*-tubocurarine (95, 125). The high activity of the erythrina alkaloids (43) shows that high curariform activity can occur in a compound which has no fully saturated quaternary nitrogen atom; it would seem that nicotine, cytisine, lobeline and other tertiary compounds (13) can have curariform actions in spite of the fact that they do not contain any quaternary nitrogen atoms. As far as we are aware all curariform compounds are either positively charged onium ions or can become such by taking up a hydrogen ion. It is possible, therefore, that the curariform activity of tertiary amines is due to their ionized forms. A study of the literature suggests that most information about the problem is available in the case of nicotine.

The blocking action of nicotine on neuromuscular transmission is well known (56, 58, 88); its action is reversible, and since the time of Langley (88) it has been known that it could block neuromuscular transmission in concentrations which left the muscle and its nerve functionally responsive to stimulation. Koppanyi, Dille and Linegar (85) showed that the ganglionic blocking action of nicotine was antagonized by physostigmine; its blocking action on the isolated rat diaphragm can also be antagonized by physostigmine (82). The available evidence, therefore, suggests that it has, in part at least, a curariform action.

Structurally nicotine is pyridyl-N-methylpyrrolidine with two dissociating groups which can take up one hydrogen ion each as follows:

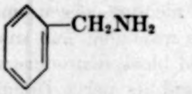


Vickery and Pucher (122) give the $pK_a$ of the methylpyrrolidine nitrogen as 8.11 and that of the pyridyl nitrogen as 3.22, both at 20°C. At physiological pH, therefore, nicotine will exist chiefly as the methyl pyrrolidine ion (b), and no appreciable quantity of the doubly charged ion (c) will be present, while a few nonionized molecules will occur. In view of the fact that the great majority of known curariform drugs are quaternary ions, it is necessary to examine the possibility that it is the monoquaternary nicotine ion which is active.

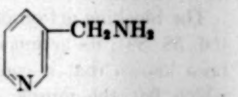
If the activity depends on the amount of the ion (b) present, its potency when assayed "in vitro" should be diminished by a rise in pH at the site of action. The diminution in potency should parallel the decrease in the concentration of (b) as the pH is raised. Since uncharged molecules diffuse faster than ions, only equilibrium methods of assay will give significant data; moreover, the influence of changes of pH on the sensitivity of the preparation to drugs of this type should be controlled by assaying the nicotine against a non-pH-sensitive fully ionized quaternary. Experiments of this type do not seem to have been done as yet. The dissociation constant of the methylpyrrolidine nitrogen atom determines the relative amounts of quaternary nicotine ions and uncharged molecules present at physiological pH values. It follows therefore that irrespective of which of these forms is active, those structural features responsible for the value of the dissociation constant of the methylpyrrolidine nitrogen atom should be considered. The work of Craig and Hixon (42) has provided us with some facts which bear on this problem. They point out that methylpyrrolidine itself is a much stronger base than it is when joined to pyridine in nicotine and that this difference is due to the intense electron attraction of pyridine. That this is indeed the correct interpretation, they confirmed by comparing the dissociation constant of the simple aliphatic methylamine with that of phenylmethylamine and β-picolylamine.

$CH_3NH_2$

$K_b = 43.8 \times 10^{-5}$



$K_b = 2 \times 10^{-5}$



$K_b = 1.1 \times 10^{-6}$

The progressive weakening of the base strength in these compounds demonstrates the increased inductive effect of these two substituents on the dissociation constant of the amino group. The electron draw of the pyridyl group in nicotine can therefore be regarded as considerably greater than that of benzene and probably sufficient to account for the pyrrolidine N dissociation constant in nicotine and hence the presence of nonionized nicotine molecules at physiological pH values.

It would appear from these considerations that the dissociation constant of the methylpyrrolidine nitrogen atom was of importance in nicotine; it possibly is, but the fact that many tertiary amines with similar pK values (morphine pK = 8.07 and codeine pK = 8.15) (32) do not have marked nicotine-like blocking actions indicates that, if it is, there must be other important factors also. The whole question of the nature of the pharmacological action of nicotine, of the effects of the pH changes on it, and of the dissociation constants of tertiary amines with actions like nicotine requires careful experimental and theoretical analysis.

There is an important and well-known effect which renders the relationship of dissociation constant to pharmacological action subject to error. It is inherent in the nature of the Van der Waals and ionic forces which are believed to assist in the fixing of drugs by receptors. The proximity of an ion to a molecule can induce the development of an opposite charge to itself on the molecule by polarizing it. Similarly, the proximity of two molecules can result in mutual polarization which, in turn, is responsible for Van der Waals forces of attraction. The proximity of a secondary or tertiary nitrogen atom to an ionic receptor would alter its dissociation constant by an amount which would depend on the polarizability of the N atom and its distance of closest approach to the polarizing charge. Considerations of this type render the previous discussion of the dissociation constants of nicotine subject to correction. Nevertheless, the dissociation of such a bound molecule should be a function of the pH of its environment, and the influence of pH on the activity of nicotine might therefore repay investigation.

The fact that these compounds are tertiary amines confers a considerable mobility on them, for many structures which are impermeable to a quaternary ion can be penetrated. This ease of penetration is responsible for the fact that, unlike curare and other quaternaries, some of these tertiary compounds are active when administered by mouth.

In the case of the quaternaries, considerable success has resulted from putting two or more quaternaries into the same molecule. The problem in the case of the tertiary amines is unlikely to yield to the same simple treatment. The difficulty can be understood by considering the dissociation constants of a dicarboxylic acid such as succinic acid. In spite of the fact that it is symmetrical and its two carboxyl groups are the same, it has two quite different dissociation constants. This is due to the fact that as soon as one carboxyl group ionizes, its acquired polarity reduces the tendency of the other to do likewise. The synthesis of a ditertiary amine would, therefore, result in its having two separate dissociation constants; if one were optimal, the other could not be. There would not seem to be any theoretical objection to a molecule having both an active full quaternary and an active reversibly dissociating tertiary nitrogen atom in it.

### *Methods of Assay*

Methods of assay of curare and curariform drugs have recently been comprehensively reviewed (15, 29, 120). The different requirements in this field, depending on the problem to be solved, and the unsatisfactory nature of most of the methods available do not, in the author's opinion, warrant any recommendations for standardization of procedure at this stage. It is doubtful if the methods as a whole have received the theoretical and experimental attention which their importance merits. The impossibility of extrapolating results obtained on animals directly to man (118), the species variation in response to drugs of this type (106), and the difficulty of designing experiments to measure true curariform activity uncomplicated by such factors as diffusion, distribution and excretion complicate the problem. In an attempt to clarify discussion, the possible purposes to which assays might be put have been listed as follows:

1. To determine the suitability of a new drug for use as a muscle relaxant in man.
2. To ensure uniformity of potency in a manufacturer's product.
3. As a screening method in the study of new drugs.
4. As a fundamental research tool in studying structure-action relationships in curariform drugs in general.

This classification is not meant to be comprehensive, for other needs might occur; nor could each section be mutually exclusive, since any problem may demand multiple methods. While the ultimate test of a curariform drug for use in clinical practice is a careful clinical trial, the requirements of an assay for use in basic research into structure-action relationships will be quite different. On this account, we will confine our attention to a few remarks about the latter.

The weight of evidence is that curare acts by reversibly combining with a tissue receptor. It was shown (70) in the case of the isolated rat diaphragm that the paralysis rate was determined by the rate at which curare could diffuse to its site of action. Any assay, therefore, which uses paralysis rates either directly or indirectly will be measuring effects, part of which at least will be determined by the diffusion rates of the drug in question. As long as a method is used to determine the concentration of a drug by assaying it against a known concentration of the same drug so that they both give the same submaximal effect, the entry of a diffusion rate limiting reaction into the assay should not matter, for the diffusion constants and gradients of both drug and standard will be the same. From Thovert's equation, we know that in simple diffusion the diffusion constants of molecules, especially the larger ones, are approximately proportionate to the reciprocal of the square roots of their molecular weights. It follows, therefore, that if we assay one drug against another by measuring paralysis rates, we will have an error in the result which is a function of the ratio of the square roots of the molecular weights of the two drugs in question.

In theory, curariform drugs should be assayed only by equilibrium methods, that is, by determining what concentration of the unknown would produce a steady state of partial block, the same as that produced by a known dose of the standard. In practice, in the intact animal it is not possible to set up precise conditions of this type, and we have to accept close approximations. The only available isolated mammalian muscle employed for assay purposes is the rat diaphragm (25) and, while it can be used to set up steady states of partial block, it has two serious disadvantages. In the first place, it is a muscle of respiration, and the ideal to be aimed at in a curariform drug is one which spares muscles of respiration while exerting marked activity on other skeletal muscle. Secondly, the resistance of rat muscle in general to drugs of the $C_{10}$ type is unusually high (106).

## SUMMARY

It is obvious that the synthesis of a safer and better muscle paralysant than *d*-tubocurarine would be a useful contribution to medicine. In spite of the simplicity of structure of many of the compounds with actions like *d*-tubocurarine and in spite of their rather localized and specific effects, our knowledge of the relation between structure and action in these compounds is far from satisfactory. The need for more information on the mode of action of *d*-tubocurarine and allied drugs and for more specific methods of assay is clear One of the main objects of this review is to render some of the other gaps in our knowledge of these and related problems more obvious

The most active curariform agents contain nitrogen atoms and these occur most commonly as fully ionized quaternaries but are also found as active tertiary atoms There is evidence that diquaternaries are, in general, more active than monoquaternaries. Complex factors associated with molecular fit have not been elucidated and it would seem that investigation of this problem will continue to be empirical for some time. There are suggestions that positive charge delocalization in onium ions reduces activity. Blocking activity in compounds containing tertiary nitrogen atoms is very interesting because in several instances it is associated with high potency. In spite of evidence that drugs showing curariform activity are usually positively charged ions, it has not yet been experimentally proven that acceptance of a proton by a tertiary nitrogen atom is necessary for it to have curariform activity. There is some theoretical reason to believe that it may be difficult to prepare a molecule having two tertiary nitrogen atoms both of which are highly active. Since there does not appear to be any similar objection to a molecule containing a full quaternary and an active tertiary nitrogen atom, study of such compounds might be profitable.

It is a pleasure to express my indebtedness to Dr. Gordon Alles for the benefit of his discussion on many points in connection with curariform compounds.

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